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**The Bioreactor Market: Steady Growth Expected**

The worldwide market for all bioreactors was valued at \$275 million for 1997, and is expected to be worth \$380 million by 2002.

1997 1997 2002  
\$275 million \$380 million

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# GENETIC ENGINEERING NEWS

GEN

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## Pharmagene Raises More Capital for Research on Human Tissues

By Sophia Fox

**P**harmagene, the Royston, U.K.-based biopharmaceutical company specializing in the use of human biomaterials for drug discovery research, has raised a further £5 million from a group of investors led by 3i and Abacus Nominees. The funding will enable the company to expand both its human biomaterials collection and its capabilities across a range of proprietary platform technologies.

Gordon Baxter, Ph.D., Pharmagene's cofounder and chief operating officer, claimed, "by the end of this year Pharmagene will have access to the largest collection of human RNAs and proteins anywhere in the world, and a range of innovative, yet robust technologies

SEE PHARMAGENE, P. 9

## Perkin-Elmer Acquires PerSeptive to Expand Its Capabilities in Gene-Based Drug Discovery

By John Sterling

**P**erkin-Elmer's (PE; Norwalk, CT) decision last month to acquire PerSeptive Biosystems (Framingham, MA) via a \$360 million stock swap was designed to strengthen PE in terms of broad capabilities in gene-based drug discovery. The company's main goal is to develop new products to improve the integration of genetic and protein research.

"This merger will enhance our position as an effective provider of innovative, integrated platforms enabling our customers to be more efficient and cost-effective in bringing new pharmaceuticals to market," says Tony L. White, PE's chairman, president and CEO. "The combination of our two companies should bolster our presence in the life sciences, [and it is our] belief that we must take bold action now to lead the emerging era of molecular medicine with leading positions in both genetic and protein analysis."

A driving force behind the merger is the vast amount of genet-



Perkin-Elmer acquired PerSeptive Biosystems for \$360 million to obtain new technologies in mass spectrometry, bioseparations and purification for product development projects, spanning the range from genomics to proteomics.

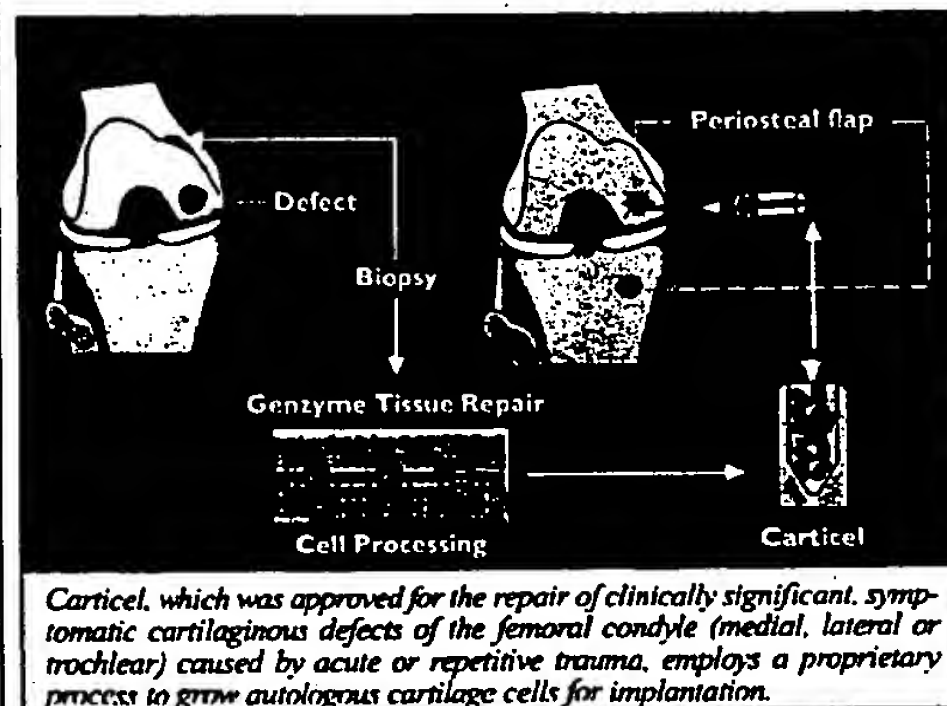
ic information about human disease that is being accumulated by researchers and biotech companies working in the area of genomics. It is becoming increasingly obvious that these data need to be complemented with technologies for

studying proteins and protein networks—a field known as proteomics (see GEN, September 1, 1997, p.1).

PE officials, who claim that MALDI-TOF (Matrix Assisted Laser Desorption/Ionization) technology is a key to

SEE ACQUISITION, P. 10

## FDA OKs Genzyme's Carticel Product for Damage to Knees



By Naomi Pfeiffer

**T**he FDA has approved a knee-cartilage replacement product made by Genzyme Tissue Repair (Cambridge, MA), a tracking-stock division of Genzyme Corp., for people with trauma-damaged knees.

Carticel (autologous cultured chondrocytes) is the first product to be licensed under the FDA's pro-

SEE GENZYME, P. 6

## Strategies for Target Validation Streamline Evaluation of Leads

By Vicki Glaser

**A**caria Biosciences (Richmond, CA) last month announced its first agreement with a major pharmaceutical company, signing a deal with Eli Lilly (Indianapolis, IN) to use Acacia's Genome Reporter Matrix (GRM) to select and optimize some of Lilly's lead compounds. Acacia's yeast-based system for profiling drug activity is useful for evaluating the therapeutic potential of lead compounds, and it also has a role in the identification and validation of new drug targets.

"We're using the ecosystem of a cell to allow us to deduce the mechanism of action and target for any chemical," explains Bruce Cohen, president and CEO. "We screen for every target in a cell simultaneously...using transcription as a readout

for how a cell is adapting to any perturbation," he says.

The GRM technology consists of two main databases: one is the genetic response profile, showing the effects of mutations in each individual yeast gene and compensatory gene regulatory mechanisms; the other is the chemical response profile, which documents changes in gene expression in response to chemical compounds. Computational analysis and pattern matching between the genetic and chemical profiles yields information on the specificity, potency and side-effects risk of a drug lead.

### Targeting Targets

No longer is mapping and sequencing a gene—or the human genome—an end unto itself, but

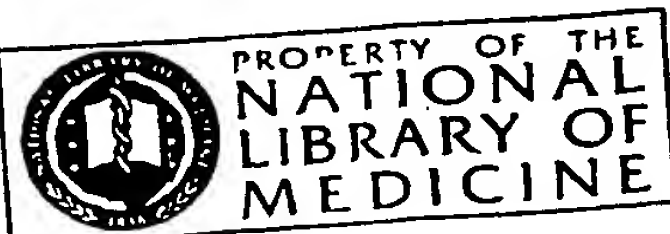
SEE TARGET, P. 15

## Sticky Ends

Avigen received two grants from the NIH & University of California for research on gene therapy for treatment of cancer & HIV infections...MRL Pharmaceutical Services, of Reston, VA, launched the TSN Bug Finder, which is able to locate & retrieve client-specified microorganisms in real-time...Gensia Sicor, Inc. will move its corporate staff from San Diego to Irvine, CA, by end of year...

FDA accepted NDA from Sepracor for levalbuterol HCl inhalation solution...An \$11.7M mezzanine financing has been closed by Activated Cell Therapy, which changed its name to Dendreon Corporation...Astra AB will build major research facility in Waltham, MA, and is also relocating Astra Arcus research facility from Rochester to Boston area...Prolifix Ltd. team used a small peptide to inhibit the E2F protein complex and induced

apoptosis in mammalian tumor cells...Ver-tex Pharmaceuticals, Inc. and Alpha Therapeutic Corp. ended an agreement to develop VX-366 for treatment of inherited hemoglobin disorders...Navi-Cyte received Phase I SBIR grant for up to \$100,000 from NIH for development of prototype of its NaviFlow technology for high-throughput screening...Covance Inc. will invest \$21 million in expansion and renovation of its facility in Indianapolis, IN.



# Target

from page 1

merely a means to an end. The critical next step is to validate the gene and its protein product as a potential drug target. The Human Genome Project continues to produce a treasure chest of expressed sequence tags (ESTs) and a tantalizing array of complete gene sequences.

Companies are applying a variety of functional genomic strategies to link genes to specific diseases and to multigenic phenotypes. Yet the ultimate challenge for pharmaceutical companies is to sift through all the sequence and differential gene expression data to identify the best targets for drug discovery.

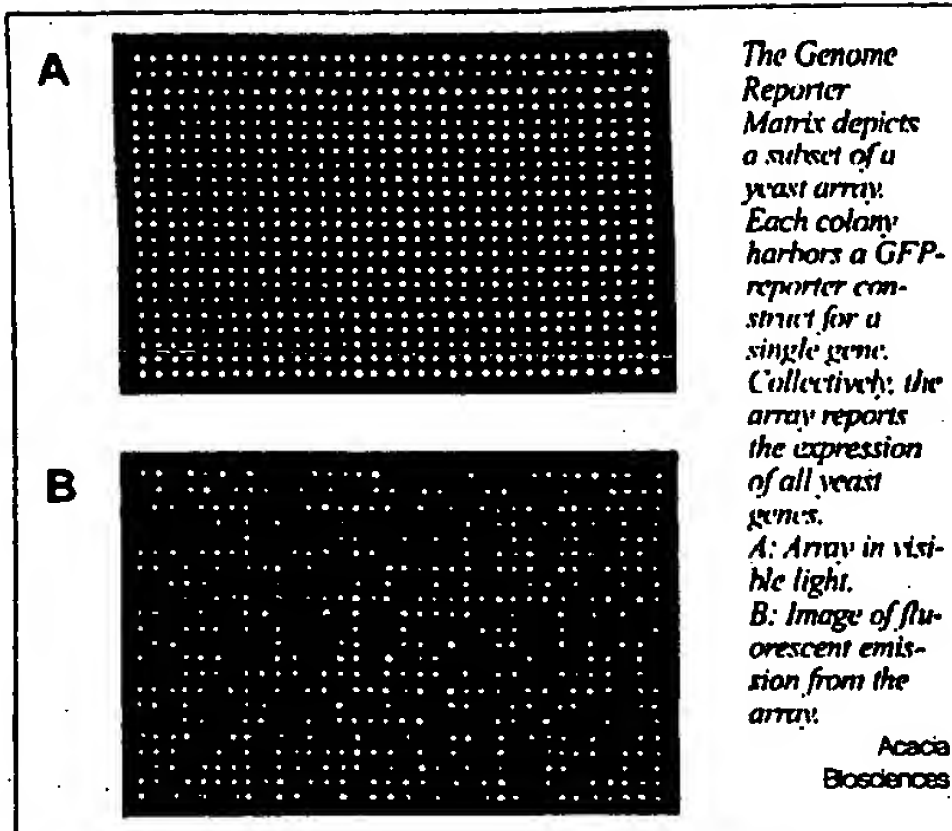
Spinning off technology developed at the University of North Carolina (Chapel Hill), Cytogen Corp. (Princeton, NJ) formed its wholly owned subsidiary AxCell Biosciences earlier this year. The young company is building a protein interaction database, cataloging all the interactions the modular domains of proteins can engage in with a

range of ligands, in order to gain insight into protein function and to select the most critical interaction to target for drug development.

AxCell's cloning-of-ligand-targets (COLT) technology employs "recognition units" from the company's genetic diversity library (GDL) to map functional protein interactions and quantitate their affinity. The company's inter-functional proteomic database (IFP-dbase) elucidates protein interaction networks and structure-activity relationships based on ligand affinity with protein modular domains.

## Defining Disease Pathways

Signal Pharmaceuticals, Inc.'s (San Diego, CA) integrated drug target and discovery effort is based on mapping gene-regulating pathways in cells and identifying small molecules that regulate the activation of those genes. In collaboration with academic researchers, the company has identified a large number of regulatory proteins in several mitogen-activated protein (MAP) kinase pathways (including the JNK, FRK and p38



**The Genome Reporter Matrix** depicts a subset of a yeast array. Each colony harbors a GFP-reporter construct for a single gene. Collectively, the array reports the expression of all yeast genes.  
A: Array in visible light.  
B: Image of fluorescent emission from the array.

Acacia Biosciences

signaling pathways), which Signal is evaluating for the treatment of autoimmune, inflammatory, cardiovascular and neurologic diseases, and cancer. Other target identification

programs focus on the NF- $\kappa$ B pathway, estrogen-related genes and central/peripheral nervous system genes.

Regulating cytokine production in immune and inflammatory disorders,

and modifying bone metabolism to treat osteoporosis are the focus of Signal's collaboration with Tanabe Seiyaku (Osaka, Japan). Signal has partnered with Organon/Akzo Nobel (Netherlands) to identify estrogen-responsive genes as targets for treating neurodegenerative and psychiatric diseases, atherosclerosis and ischemia, and with Roche Bioscience (Palo Alto, CA) to develop human peripheral nerve cell lines for the discovery of treatments for pain and incontinence.

Exelixis' (S. San Francisco, CA) strategy for target selection is to define disease pathways and identify regulatory molecules that activate or inhibit those biochemical/genetic pathways. Based on the finding that these pathways are conserved across species, the company is studying the model genetic systems of *Drosophila* and *Caenorhabditis elegans*. Using its PathFinder technology, Exelixis systematically introduces mutations into the genomes of these model organisms, looking for mutations that enhance or suppress the target disease-related gene. These novel genes then become the basis of drug screening assays.

Cadus Pharmaceutical Corp. (Tarrytown, NY) is identifying surrogate ligands to newly discovered orphan G-protein coupled transmembrane receptors of unknown function to determine the suitability of the receptors as drug targets. Inserting the novel receptor in a yeast system yields a ligand that activates the receptor. Access to a surrogate ligand allows the company to screen for receptor antagonists in the yeast system.

"The antagonist plus the surrogate ligand gives you two probes—an on probe and an off probe—which allows you to look at function," explains David Webb, Ph.D., vp of research and chief scientific officer. A surrogate ligand also provides information on which G-protein interacts with the orphan receptor and its associated signaling pathways, further clarifying the role of the receptor as a potential drug target. Cadus' collaboration with SmithKline (Philadelphia) capitalizes on Cadus' ability to determine orphan receptor function, applying the technology to SmithKline's proprietary, newly discovered G-protein receptors.

Cadus' recombinant yeast system can also be used to screen cell and tissue extracts for natural ligands, and the company is accelerating its internal drug-discovery efforts in the areas of cancer, inflammation and allergy. A recent equity investment in Axiom Biotechnologies (San Diego, CA) gave Cadus a license to Axiom's high-throughput pharmacologic screening system for lead optimization and discovery.

As its name implies, gene/Networks (Alameda, CA) focuses on identifying gene networks that contribute to multigenic phenotypes and complex disease processes. The integration of mouse and human genetic studies forms the basis of the technology. The Genome Tagged Mice database in development will serve as a library of natural mouse genetic and phenotypic variation. Disease-related genes identified in mice are then evaluated in human family- and population-based studies to confirm their clinical relevance and linkages to pathophysiological traits.

## Blocking Gene Expression

Inactivating a gene known to be expressed in association with a particular disease is one approach to identifying appropriate therapeutic targets. The target validation and discovery program at Ribozyme Pharmaceuticals, Inc. (Boulder, CO) applies the company's ribozyme technology to achieve selective inhibition of gene expression in cell culture and in animals.

Correlation of the gene expression inhibition with phenotype can

SEE TARGET, P. 38

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## Target

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suggest the relative importance of the gene in disease pathology. The company's nuclease-resistant ribozymes form the basis of a collaboration with Schering AG (Germany) for drug target validation and the development of ribozyme-based therapeutic agents, and with Chiron Corp. (Emeryville, CA) for target validation.

With several antisense compounds now progressing through clinical trials, the concept of using oligonucleotides to inhibit gene activity is not new. But rather than focusing on therapeutics development, Sequitur, Inc. (Natick, MA) is creating antisense compounds for the purpose of determining gene function and validating drug targets. Clients typically provide the one-year-old company with the sequence (or EST) of a potential gene target and, in return, Sequitur custom designs a series of three to six antisense compounds that yield a three-to-ten-fold inhibition of the target gene in cell culture. The company also provides oligonucleotides, a series of cationic lipids, to deliver the oligonucleotides to a variety of cultured cells.

"Differential expression information is just for correlation, it doesn't tell function or confirm what would be a good target," says Tod Woolf, Ph.D., director of technology development at Sequitur. Whereas, antisense compounds will inhibit a target, Sequitur offers both phosphorothioate DNA antisense compounds, and its proprietary Next Generation chimeric oligonucleotides, which have a higher hybridization affinity, greater specificity and reduced toxicity, according to the company.

### Mining Pathogen Genomes

Companies such as Human Genome Sciences (HGS; Rockville, MD), Incyte (Palo Alto, CA),

*AxCell Biosciences scientists say their technology enables the rapid and simple functional identification of the two essential molecular components of protein interaction networks: specific recognition units that bind distinct modular protein domains are identified and isolated using a combination structural/functional approach that uses both peptide phase display Genetic Diversity Libraries (GDL) and bioinformatics, and cloning of Ligand Targets (COLT) technology utilizes recognition units as functional probes to isolate families of interactor proteins.*

Millennium Pharmaceuticals Inc. (Cambridge, MA) and Genome Therapeutics (Waltham, MA) are relying on high-speed DNA sequencing, positional cloning and other strategies to identify specific microbial genomic sites that would be good targets for infectious disease therapeutics.

HGS recently completed sequencing of the bacterial pathogen *Streptococcus pneumoniae*, which is the focus of an agreement with Hoffmann-La Roche (Basel, Switzerland). Roche will use the sequence data to develop new anti-infectives against *S. pneumoniae*. HGS and Roche have expanded their collaboration to include a nonexclusive license to access sequence information for the intestinal bacterium *Enterococcus faecalis*.

Incyte Pharmaceuticals has completed one-fold coverage of the *Candida albicans* genome, identify-

ing 60% of the genes of this fungal pathogen. This genome will become part of the company's PathoSeq microbial database. Incyte recently introduced the ZooSeq animal gene sequence and expression database. The database will provide genomic information across various species commonly used in preclinical drug testing, which may help to better define potential drug targets.

Millennium Pharmaceuticals continues to report success in identifying novel drug targets, having recently discovered a novel chemokine called neurotactin and a new class of MAD-related proteins that inhibit transforming growth factor beta (TGF- $\beta$ ) signaling. The company also received U.S. patent coverage for the tub genes, believed to play a role in obesity, and for the gene that encodes the protein melastatin, which appears to suppress metastasis in malignant melanoma.

## Pangea

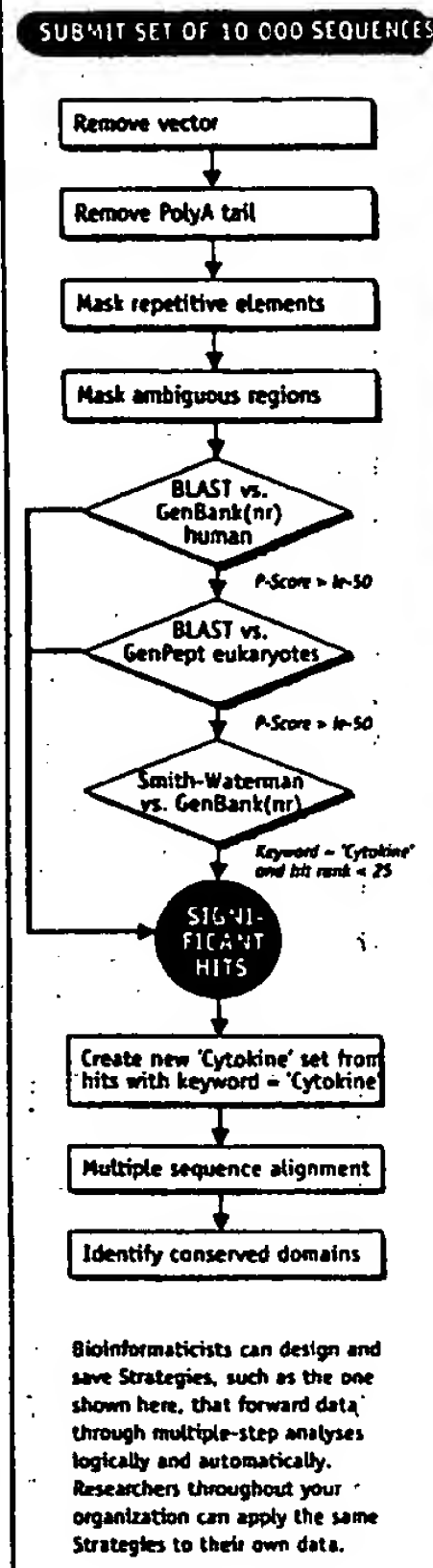
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Smith, now a computer programmer, is an expert in systems integration, Internet technologies and the application of industrial engineering principles to the drug discovery process. Before co-founding Pangea, he was the manager of software development at Attorney's Briefcase, a legal research software company.

By being "in the trenches" with customers and collaborators, Bellenson and Smith sensed the frustration of pharmaceutical researchers whose incompatible tools have impeded their progress. According to Bellenson, "Most of them are geared toward analyzing one molecule at a time. It's like emptying the ocean with an eye dropper—an incompatible eye dropper at that. A pharmaceutical company may have 30 different drug discovery teams with various approaches. The problem is to manage the process of experimenting with a lot of different approaches, to automate while maintaining flexibility."

GeneWorld 2.1 enables "integration of the entire target discovery and validation process," Bellenson says. The commercial software package coordinates the entire process of sequence-data analysis and can be integrated with other programs and databases, according to Smith, who adds that it handles thousands of sequence results, organizes and automates annotation and seamlessly interacts with growing genome databases. Simple forms and menus enable users to turn raw sequence data into crucial knowledge for drug discovery by applying algorithms to sequences, creating custom analysis strategies and producing useful reports, without the need for writing computer code. GeneWorld 2.1 runs on a variety of platforms and operating systems.

Pairing industrial relational database-management systems with a web-browser interface, Pangea's Operating System of Drug Discovery is an open-computing framework that allows client/server and Java-enabled web-based technologies to collect, organize and analyze drug discovery information for pharmaceutical companies to simplify and accelerate drug discovery. The technology unites automated genomics database analysis for drug target site selection, chemical information database analysis and large-scale combinatorial chemistry project management and high-throughput screening project management for drug lead efficacy analysis. Pangea officials maintain that these integrated elements provide a unified environment for chemists, biologists and others involved in the drug discovery process to work together with



commercial and public domain software.

Pangea's Operating System of Drug Discovery can accommodate Sybase, Oracle or Informix relational database-management systems and any version of UNIX. It absorbs new data formats, databases, algorithms and analysis paradigms into the automated workflow without software modifications. Netscape Navigator provides a friendly user interface from PC, Macintosh, and UNIX workstations.

In the near term, Pangea plans to complete its bioinformatics core with two more programs. Gene Foundry, a sample tracking and workflow sequence package for DNA sequence and fragment information, will also offer interaction with robots, reagent tracking and troubleshooting. Gene Thesaurus, the other package is a "warehouse of bioinformatics data," says Bellenson.

## Europe

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GTAC Chairman, Professor Norman C. Nevin, said 1996 saw "four important developments": an increase in enquiries and submissions made to GTAC; an increase in the complexity of submitted protocols; a continuing shift from gene therapy for single-gene disorders toward strategies aimed at tumour destruction in cancer; and a growth in international sponsorship of U.K. gene therapy trials.

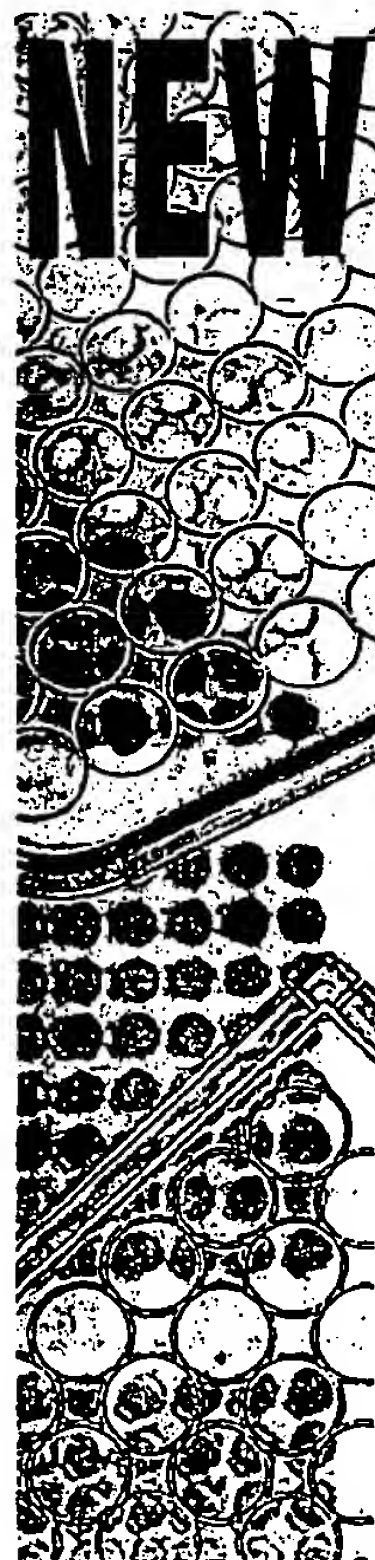
Since 1993, GTAC and its predecessor, the Clothier Committee, have approved 18 U.K. gene therapy clinical trials (13 of which have been carried out), which are listed in the report. The disease areas targeted by these trials include severe combined immunodeficiency (1 trial), cystic fibrosis (6), metastatic melanoma (2), lymphoma (2), neuroblastoma (1), breast cancer (1), Hurler's syndrome (1), cervical cancer (1), glioblastoma

breast cancer, breast cancer with liver metastases, glioblastoma, malignant ascites due to gastrointestinal cancer and ovarian cancer.

Copies of the GTAC third annual report are available from the GTAC Secretariat, Wellington House, 133-155 Waterloo Road, London SE1 8UG, U.K.

### Coated Lenses Prevent PCO

Scientists in the U.K. say it may be possible to prevent posterior capsule opacification (PCO), a common complication following cataract surgery, by using the implanted polymethylmethacrylate (PMMA) intraocular lens as a drug delivery system. PCO occurs in 30-50% of cataract surgery patients as a result of stimulated cell growth within the remaining capsular bag. The condition causes a decline in visual acuity and requires expensive laser treatment, thus negating the routine use of cataract surgery in underdeveloped countries, explains G. Duncan, at the



## NEW HIGH SPECIFIC ACTIVITY MICROBIAL ALKALINE PHOSPHATASE from Biocatalysts

Biocatalysts Limited, the British speciality enzyme company, has developed a completely new type of alkaline phosphatase with many advantages over the types most commonly used.

It is of microbial origin with a high specific activity (unlike that from *E. coli*) and with higher temperature and storage stability compared to that from calf intestine.

This is the first of several new generation diagnostic enzymes being developed by Biocatalysts Limited with greatly improved stability.

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For further details on alkaline phosphatase and our other diagnostic enzymes contact us direct at the address below or within North America contact our US Distributor Kaltron-Pettibone 'phone: 630 350 1116 or fax: 630-350-1606

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Tel: +44 (0)1443 843712 Fax: +44 (0)1443 841214  
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28 April 1997; accepted 9 September 1997

## Exploring the Metabolic and Genetic Control of Gene Expression on a Genomic Scale

Joseph L. DeRisi, Vishwanath R. Iyer, Patrick O. Brown\*

DNA microarrays containing virtually every gene of *Saccharomyces cerevisiae* were used to carry out a comprehensive investigation of the temporal program of gene expression accompanying the metabolic shift from fermentation to respiration. The expression profiles observed for genes with known metabolic functions pointed to features of the metabolic reprogramming that occur during the diauxic shift, and the expression patterns of many previously uncharacterized genes provided clues to their possible functions. The same DNA microarrays were also used to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*. These results demonstrate the feasibility and utility of this approach to genomewide exploration of gene expression patterns.

The complete sequences of nearly a dozen microbial genomes are known, and in the next several years we expect to know the complete genome sequences of several metazoans, including the human genome. Defining the role of each gene in these genomes will be a formidable task, and understanding how the genome functions as a whole in the complex natural history of a living organism presents an even greater challenge.

Knowing when and where a gene is expressed often provides a strong clue as to its biological role. Conversely, the pattern of genes expressed in a cell can provide detailed information about its state. Although regulation of protein abundance in a cell is by no means accomplished solely by regulation of mRNA, virtually all differences in cell type or state are correlated with changes in the mRNA levels of many genes. This is fortuitous because the only specific reagent required to measure the abundance of the mRNA for a specific gene is a cDNA sequence. DNA microarrays, consisting of thousands of individual gene sequences printed in a high-density array on a glass microscope slide (1, 2), provide a practical and economical tool for studying gene expression on a very large scale (3–6).

*Saccharomyces cerevisiae* is an especially

favorable organism in which to conduct a systematic investigation of gene expression. The genes are easy to recognize in the genome sequence, cis regulatory elements are generally compact and close to the transcription units, much is already known about its genetic regulatory mechanisms, and a powerful set of tools is available for its analysis.

A recurring cycle in the natural history of yeast involves a shift from anaerobic (fermentation) to aerobic (respiration) metabolism. Inoculation of yeast into a medium rich in sugar is followed by rapid growth fueled by fermentation, with the production of ethanol. When the fermentable sugar is exhausted, the yeast cells turn to ethanol as a carbon source for aerobic growth. This switch from anaerobic growth to aerobic respiration upon depletion of glucose, referred to as the diauxic shift, is correlated with widespread changes in the expression of genes involved in fundamental cellular processes such as carbon metabolism, protein synthesis, and carbohydrate storage (7). We used DNA microarrays to characterize the changes in gene expression that take place during this process for nearly the entire genome, and to investigate the genetic circuitry that regulates and executes this program.

Yeast open reading frames (ORFs) were amplified by the polymerase chain reaction (PCR), with a commercially available set of primer pairs (8). DNA microarrays, containing approximately 6400 distinct DNA sequences, were printed onto glass slides by

using a simple robotic printing device (9). Cells from an exponentially growing culture of yeast were inoculated into fresh medium and grown at 30°C for 21 hours. After an initial 9 hours of growth, samples were harvested at seven successive 2-hour intervals, and mRNA was isolated (10). Fluorescently labeled cDNA was prepared by reverse transcription in the presence of Cy3(green)- or Cy5(red)-labeled deoxyuridine triphosphate (dUTP) (11) and then hybridized to the microarrays (12). To maximize the reliability with which changes in expression levels could be discerned, we labeled cDNA prepared from cells at each successive time point with Cy5, then mixed it with a Cy3-labeled "reference" cDNA sample prepared from cells harvested at the first interval after inoculation. In this experimental design, the relative fluorescence intensity measured for the Cy3 and Cy5 fluors at each array element provides a reliable measure of the relative abundance of the corresponding mRNA in the two cell populations (Fig. 1). Data from the series of seven samples (Fig. 2), consisting of more than 43,000 expression-ratio measurements, were organized into a database to facilitate efficient exploration and analysis of the results. This database is publicly available on the Internet (13).

During exponential growth in glucose-rich medium, the global pattern of gene expression was remarkably stable. Indeed, when gene expression patterns between the first two cell samples (harvested at a 2-hour interval) were compared, mRNA levels differed by a factor of 2 or more for only 19 genes (0.3%), and the largest of these differences was only 2.7-fold (14). However, as glucose was progressively depleted from the growth media during the course of the experiment, a marked change was seen in the global pattern of gene expression. mRNA levels for approximately 710 genes were induced by a factor of at least 2, and the mRNA levels for approximately 1030 genes declined by a factor of at least 2. Messenger RNA levels for 183 genes increased by a factor of at least 4, and mRNA levels for 203 genes diminished by a factor of at least 4. About half of these differentially expressed genes have no currently recognized function and are not yet named. Indeed, more than 400 of the differentially expressed genes have no apparent homology

Department of Biochemistry, Stanford University School of Medicine, Howard Hughes Medical Institute, Stanford, CA 94305–5428, USA.

\*To whom correspondence should be addressed. E-mail: pbrown@cmgm.stanford.edu

to any gene whose function is known (15). The responses of these previously uncharacterized genes to the diauxic shift therefore provides the first small clue to their possible roles.

The global view of changes in expression of genes with known functions provides a vivid picture of the way in which the cell adapts to a changing environment. Figure 3 shows a portion of the yeast metabolic pathways involved in carbon and energy metabolism. Mapping the changes we observed in the mRNAs encoding each enzyme onto this framework allowed us to infer the redirection in the flow of metabolites through this system. We observed large inductions of the genes coding for the enzymes aldehyde dehydrogenase (ALD2) and acetyl-coenzyme A (CoA) synthase (ACS1), which function together to convert the products of alcohol dehydrogenase into acetyl-CoA, which in turn is used to fuel the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. The concomitant shutdown of transcription of the genes encoding pyruvate decarboxylase and induction of pyruvate carboxylase rechannels pyruvate away from acetaldehyde, and instead to oxalacetate, where it can serve to supply the TCA cycle and gluconeogenesis. Induction of the pivotal genes *PCK1*, encoding phosphoenolpyruvate carboxykinase, and *FBP1*, encoding fructose 1,6-bisphosphatase, switches the directions of two key irreversible steps in glycolysis, reversing the flow of metabolites along the reversible steps of the glycolytic pathway toward the essential biosynthetic precursor, glucose-6-phosphate. Induction of the genes coding for the trehalose synthase and glycogen synthase complexes promotes channeling of glucose-6-phosphate into these carbohydrate storage pathways.

Just as the changes in expression of genes encoding pivotal enzymes can provide insight into metabolic reprogramming, the behavior of large groups of functionally related genes can provide a broad view of the systematic way in which the yeast cell adapts to a changing environment (Fig. 4). Several classes of genes, such as cytochrome *c*-related genes and those involved in the TCA/glyoxylate cycle and carbohydrate storage, were coordinately induced by glucose exhaustion. In contrast, genes devoted to protein synthesis, including ribosomal proteins, tRNA synthetases, and translation, elongation, and initiation factors, exhibited a coordinated decrease in expression. More than 95% of ribosomal genes showed at least twofold decreases in expression during the diauxic shift (Fig. 4) (13). A noteworthy and illuminating exception was that the

genes encoding mitochondrial ribosomal genes were generally induced rather than repressed after glucose limitation, highlighting the requirement for mitochondrial biogenesis (13). As more is learned about the functions of every gene in the yeast genome, the ability to gain insight into a cell's response to a changing environment through its global gene expression patterns will become increasingly powerful.

Several distinct temporal patterns of expression could be recognized, and sets of genes could be grouped on the basis of the similarities in their expression patterns. The characterized members of each of these groups also shared important similarities in their functions. Moreover, in most cases, common regulatory mechanisms could be inferred for sets of genes with similar expression profiles. For example, seven genes showed a late induction profile, with mRNA levels increasing by more than ninefold at

the last timepoint but less than threefold at the preceding timepoint (Fig. 5B). All of these genes were known to be glucose-repressed, and five of the seven were previously noted to share a common upstream activating sequence (UAS), the carbon source response element (CSRE) (16–20). A search in the promoter regions of the remaining two genes, *ACR1* and *IDP2*, revealed that *ACR1*, a gene essential for *ACS1* activity, also possessed a consensus CSRE motif, but interestingly, *IDP2* did not. A search of the entire yeast genome sequence for the consensus CSRE motif revealed only four additional candidate genes, none of which showed a similar induction.

Examples from additional groups of genes that shared expression profiles are illustrated in Fig. 5, C through F. The sequences upstream of the named genes in Fig. 5C all contain stress response elements (STRE), and with the exception

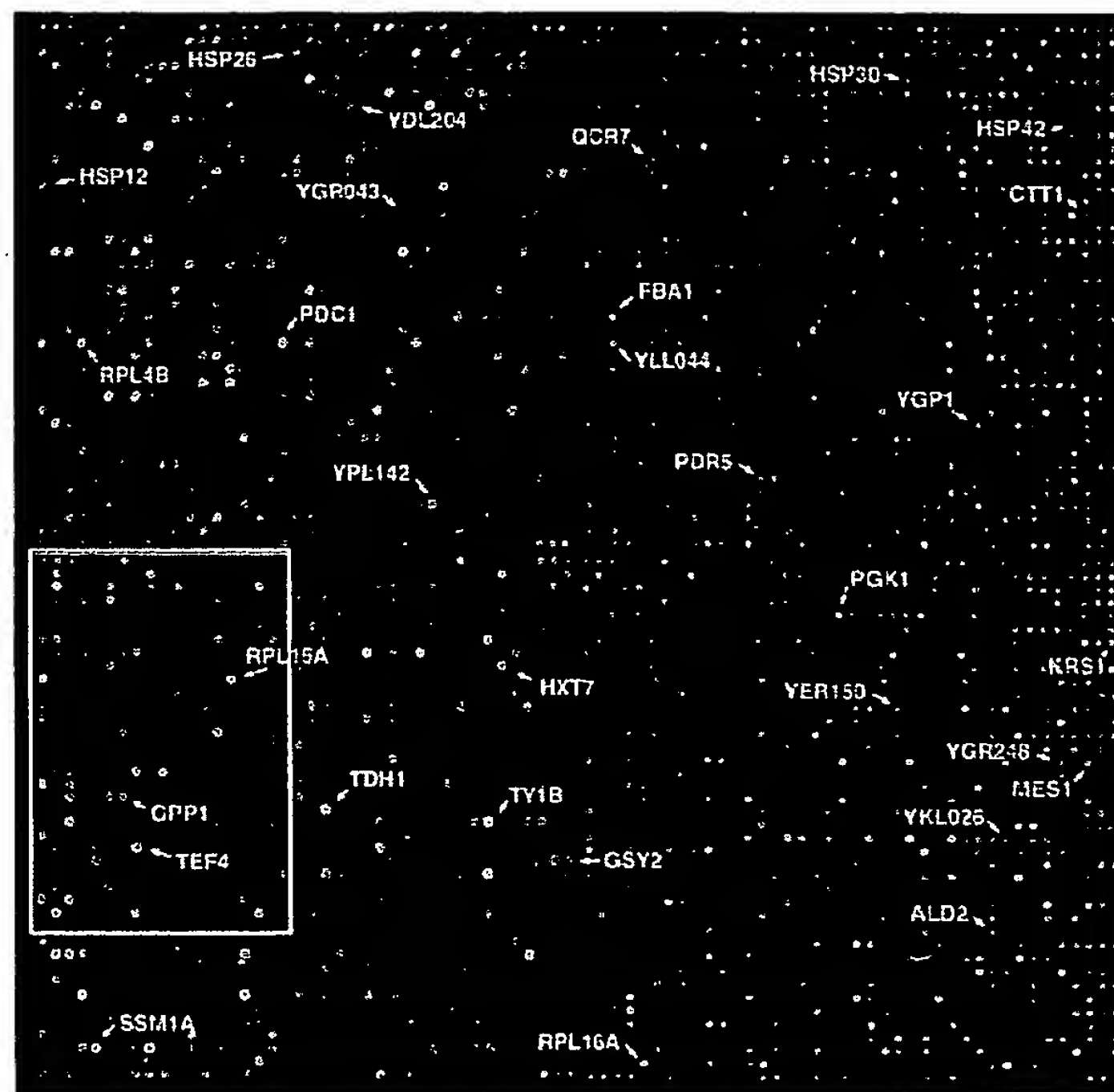


Fig. 1. Yeast genome microarray. The actual size of the microarray is 18 mm by 18 mm. The microarray was printed as described (9). This image was obtained with the same fluorescent scanning confocal microscope used to collect all the data we report (49). A fluorescently labeled cDNA probe was prepared from mRNA isolated from cells harvested shortly after inoculation (culture density of  $<5 \times 10^6$  cells/ml and media glucose level of 19 g/liter) by reverse transcription in the presence of Cy3-dUTP. Similarly, a second probe was prepared from mRNA isolated from cells taken from the same culture 9.5 hours later (culture density of  $\sim 2 \times 10^8$  cells/ml, with a glucose level of  $<0.2$  g/liter) by reverse transcription in the presence of Cy5-dUTP. In this image, hybridization of the Cy3-dUTP-labeled cDNA (that is, mRNA expression at the initial timepoint) is represented as a green signal, and hybridization of Cy5-dUTP-labeled cDNA (that is, mRNA expression at 9.5 hours) is represented as a red signal. Thus, genes induced or repressed after the diauxic shift appear in this image as red and green spots, respectively. Genes expressed at roughly equal levels before and after the diauxic shift appear in this image as yellow spots.

of HSP42, have previously been shown to be controlled at least in part by these elements (21–24). Inspection of the sequences upstream of HSP42 and the two uncharacterized genes shown in Fig. 5C, YKL026c, a hypothetical protein with similarity to glutathione peroxidase, and YGR043c, a putative transaldolase, revealed that each of these genes also possess repeated upstream copies of the stress-responsive CCCCT motif. Of the 13 additional genes in the yeast genome that shared this expression profile [including HSP30, ALD2, OM45, and 10 uncharacterized ORFs (25)], nine contained one or more recognizable STRE sites in their upstream regions.

The heterotrimeric transcriptional activator complex HAP2,3,4 has been shown to be responsible for induction of several genes important for respiration (26–28). This complex binds a degenerate consensus sequence known as the CCAAT box (26). Computer analysis, using the consensus sequence TNRYTGGB (29), has suggested that a large number of genes involved in respiration may be specific targets of HAP2,3,4 (30). Indeed, a putative HAP2,3,4 binding site could be found in the sequences upstream of each of the seven cytochrome c-related genes that showed the greatest magnitude of induction (Fig. 5D). Of 12 additional cytochrome c-related genes that were induced, HAP2,3,4 binding sites were present in all but one. Significantly, we found that transcription of HAP4 itself was induced nearly ninefold concomitant with the diauxic shift.

Control of ribosomal protein biogenesis is mainly exerted at the transcriptional level, through the presence of a common upstream-activating element (UAS<sub>rp</sub>) that is recognized by the Rap1 DNA-binding protein (31, 32). The expression profiles of seven ribosomal proteins are shown in Fig. 5F. A search of the sequences upstream of all seven genes revealed consensus Rap1-binding motifs (33). It has been suggested that declining Rap1 levels in the cell during starvation may be responsible for the decline in ribosomal protein gene expression (34). Indeed, we observed that the abundance of RAP1 mRNA diminished by 4.4-fold, at about the time of glucose exhaustion.

Of the 149 genes that encode known or putative transcription factors, only two, HAP4 and SIP4, were induced by a factor of more than threefold at the diauxic shift. SIP4 encodes a DNA-binding transcriptional activator that has been shown to interact with Snf1, the "master regulator" of glucose repression (35). The eightfold induction of SIP4 upon depletion of glucose strongly suggests a role in the induction of

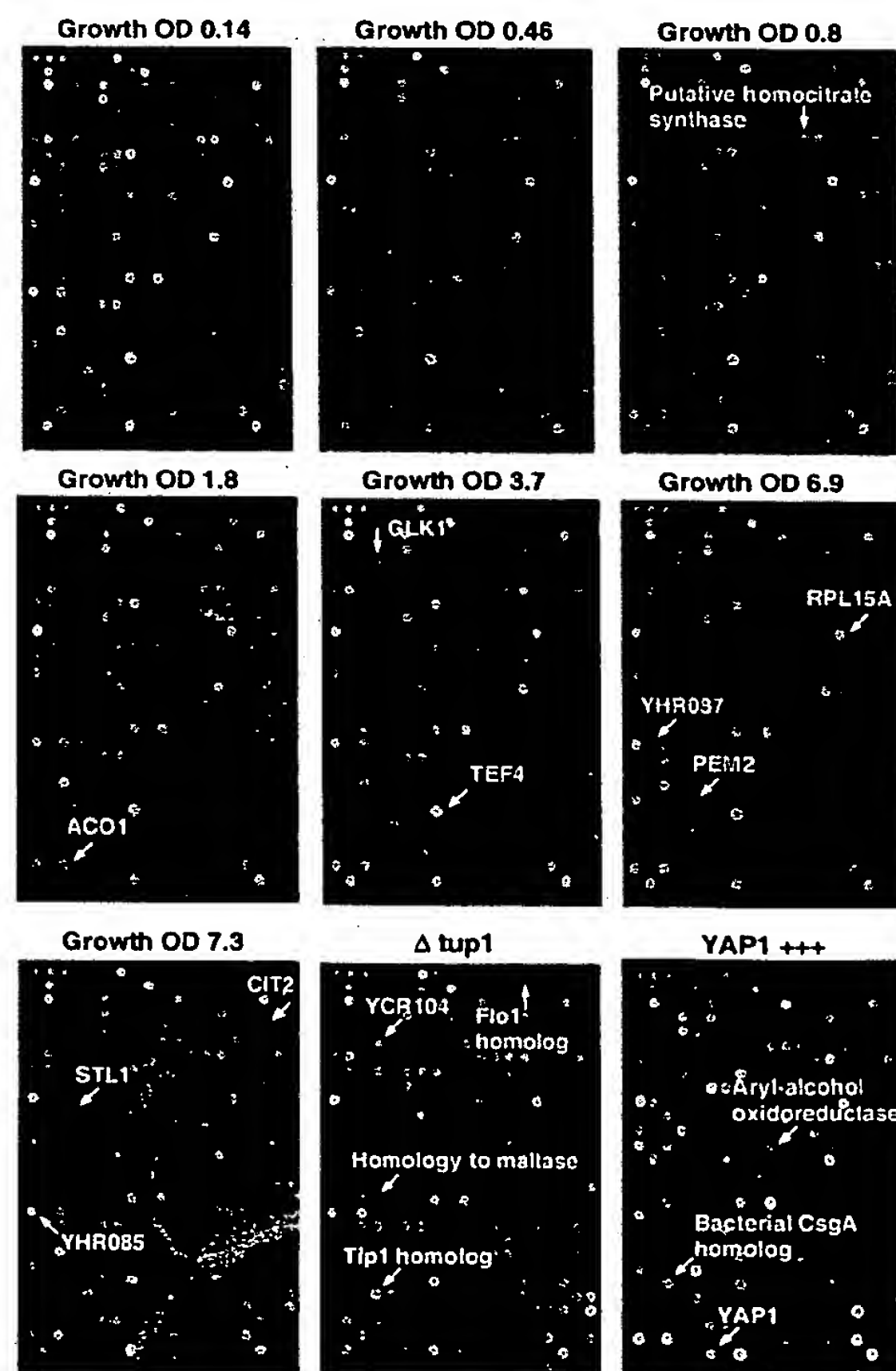
downstream genes at the diauxic shift.

Although most of the transcriptional responses that we observed were not previously known, the responses of many genes during the diauxic shift have been described. Comparison of the results we obtained by DNA microarray hybridization with previously reported results therefore provided a strong test of the sensitivity and accuracy of this approach. The expression patterns we observed for previously characterized genes showed almost perfect concordance with previously published results (36). Moreover, the differential expression measurements obtained by DNA microarray hybridization were reproducible in duplicate experiments. For example, the remarkable changes in gene expression between cells harvested immediately after inoculation and immediately after the diauxic shift (the first and sixth intervals in this time series) were measured in duplicate, independent DNA microarray hybridizations. The correlation coefficient for two complete sets of expression ratio measurements was 0.87, and for more than 95% of the genes, the expres-

sion ratios measured in these duplicate experiments differed by less than a factor of 2. However, in a few cases, there were discrepancies between our results and previous results, pointing to technical limitations that will need to be addressed as DNA microarray technology advances (37, 38). Despite the noted exceptions, the high concordance between the results we obtained in these experiments and those of previous studies provides confidence in the reliability and thoroughness of the survey.

The changes in gene expression during this diauxic shift are complex and involve integration of many kinds of information about the nutritional and metabolic state of the cell. The large number of genes whose expression is altered and the diversity of temporal expression profiles observed in this experiment highlight the challenge of understanding the underlying regulatory mechanisms. One approach to defining the contributions of individual regulatory genes to a complex program of this kind is to use DNA microarrays to identify genes whose expression is affected

**Fig. 2.** The section of the array indicated by the gray box in Fig. 1 is shown for each of the experiments described here. Representative genes are labeled. In each of the arrays used to analyze gene expression during the diauxic shift, red spots represent genes that were induced relative to the initial timepoint, and green spots represent genes that were repressed relative to the initial timepoint. In the arrays used to analyze the effects of the *tup1*Δ mutation and YAP1 overexpression, red spots represent genes whose expression was increased, and green spots represent genes whose expression was decreased by the genetic modification. Note that distinct sets of genes are induced and repressed in the different experiments. The complete images of each of these arrays can be viewed on the Internet (13). Cell density as measured by optical density (OD) at 600 nm was used to measure the growth of the culture.





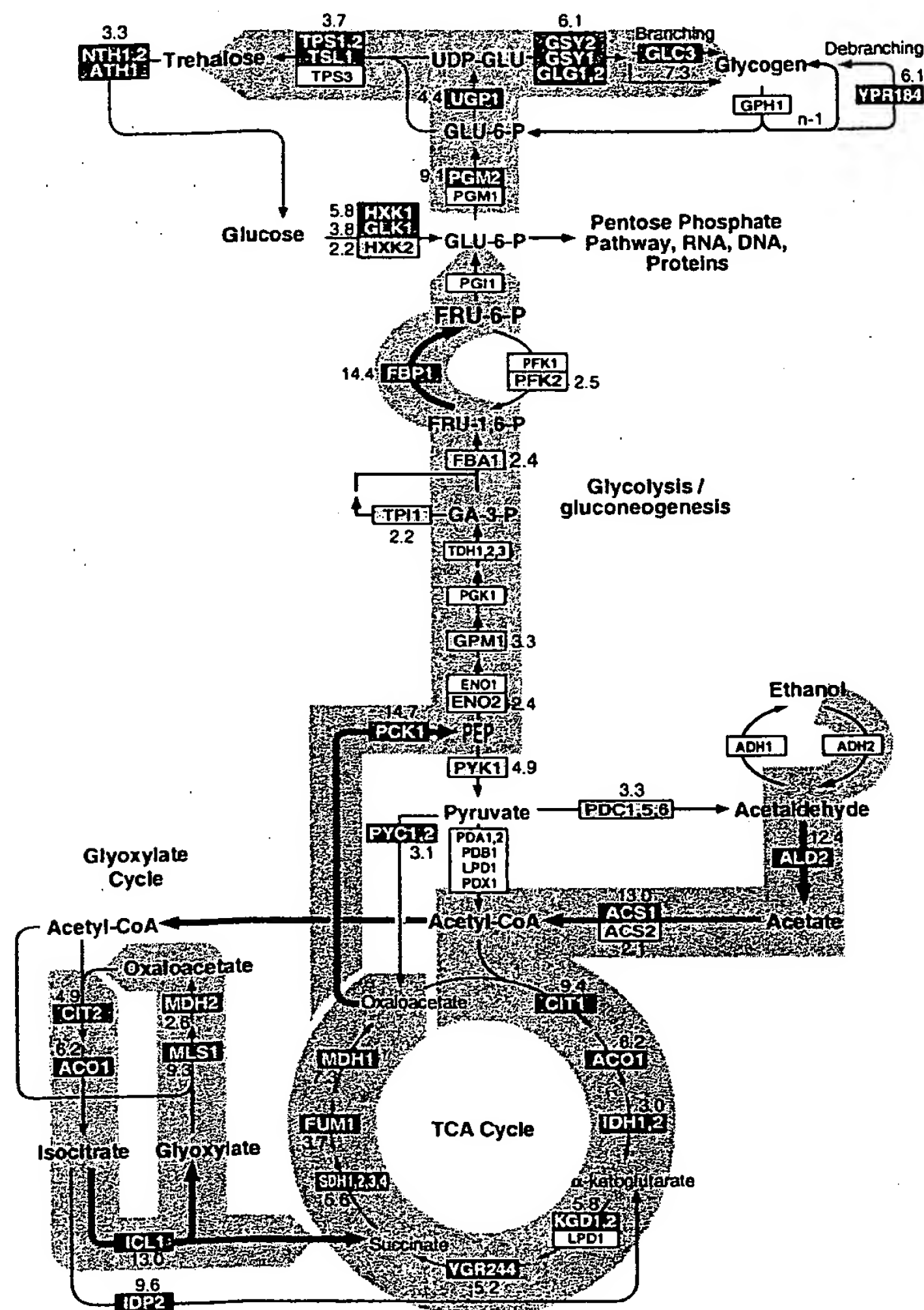
by mutations in each putative regulatory gene. As a test of this strategy, we analyzed the genome-wide changes in gene expression that result from deletion of the *TUP1* gene. Transcriptional repression of many genes by glucose requires the DNA-binding repressor

Mig1 and is mediated by recruiting the transcriptional co-repressors Tup1 and Cyc8/Ssn6 (39). Tup1 has also been implicated in repression of oxygen-regulated, mating-type-specific, and DNA-damage-inducible genes (40).

Wild-type yeast cells and cells bearing a deletion of the *TUP1* gene (*tup1Δ*) were grown in parallel cultures in rich medium containing glucose as the carbon source. Messenger RNA was isolated from exponentially growing cells from the two populations and used to prepare cDNA labeled with Cy3 (green) and Cy5 (red), respectively (11). The labeled probes were mixed and simultaneously hybridized to the microarray. Red spots on the microarray therefore represented genes whose transcription was induced in the *tup1Δ* strain, and thus presumably repressed by Tup1 (41). A representative section of the microarray (Fig. 2, bottom middle panel) illustrates that the genes whose expression was affected by the *tup1Δ* mutation, were, in general, distinct from those induced upon glucose exhaustion [complete images of all the arrays shown in Fig. 2 are available on the Internet (13)]. Nevertheless, 34 (10%) of the genes that were induced by a factor of at least 2 after the diauxic shift were similarly induced by deletion of *TUP1*, suggesting that these genes may be subject to *TUP1*-mediated repression by glucose. For example, *SUC2*, the gene encoding invertase, and all five hexose transporter genes that were induced during the course of the diauxic shift were similarly induced, in duplicate experiments, by the deletion of *TUP1*.

The set of genes affected by Tup1 in this experiment also included  $\alpha$ -glucosidases, the mating-type-specific genes *MFA1* and *MFA2*, and the DNA damage-inducible *RNR2* and *RNR4*, as well as genes involved in flocculation and many genes of unknown function. The hybridization signal corresponding to expression of *TUP1* itself was also severely reduced because of the (incomplete) deletion of the transcription unit in the *tup1Δ* strain, providing a positive control in the experiment (42).

Many of the transcriptional targets of Tup1 fell into sets of genes with related biochemical functions. For instance, although only about 3% of all yeast genes appeared to be *TUP1*-repressed by a factor of more than 2 in duplicate experiments under these conditions, 6 of the 13 genes that have been implicated in flocculation (15) showed a reproducible increase in expression of at least twofold when *TUP1* was deleted. Another group of related genes that appeared to be subject to *TUP1* repression encodes the serine-rich cell wall mannoproteins, such as *Tip1* and *Tir1/Srp1* which are induced by cold shock and other stresses (43), and similar, serine-poor proteins, the seripauperins (44). Messenger RNA levels for 23 of the 26 genes in this group were reproducibly elevated by at least 2.5-fold in the *tup1Δ*



**Fig. 3.** Metabolic reprogramming inferred from global analysis of changes in gene expression. Only key metabolic intermediates are identified. The yeast genes encoding the enzymes that catalyze each step in this metabolic circuit are identified by name in the boxes. The genes encoding succinyl-CoA synthase and glycogen-debranching enzyme have not been explicitly identified, but the ORFs YGR244 and YPR184 show significant homology to known succinyl-CoA synthase and glycogen-debranching enzymes, respectively, and are therefore included in the corresponding steps in this figure. Red boxes with white lettering identify genes whose expression increases in the diauxic shift. Green boxes with dark green lettering identify genes whose expression diminishes in the diauxic shift. The magnitude of induction or repression is indicated for these genes. For multimeric enzyme complexes, such as succinate dehydrogenase, the indicated fold-induction represents an unweighted average of all the genes listed in the box. Black and white boxes indicate no significant differential expression (less than twofold). The direction of the arrows connecting reversible enzymatic steps indicate the direction of the flow of metabolic intermediates, inferred from the gene expression pattern, after the diauxic shift. Arrows representing steps catalyzed by genes whose expression was strongly induced are highlighted in red. The broad gray arrows represent major increases in the flow of metabolites after the diauxic shift, inferred from the indicated changes in gene expression.

strain, and 18 of these genes were induced by more than sevenfold when *TUP1* was deleted. In contrast, none of 83 genes that could be classified as putative regulators of the cell division cycle were induced more than twofold by deletion of *TUP1*. Thus, despite the diversity of the regulatory systems that employ Tup1, most of the genes that it regulates under these conditions fall into a limited number of distinct functional classes.

Because the microarray allows us to monitor expression of nearly every gene in yeast, we can, in principle, use this approach to identify all the transcriptional targets of a regulatory protein like Tup1. It is important to note, however, that in any single experiment of this kind we can only recognize those target genes that are normally repressed (or induced) under the conditions of the experiment. For instance, the experiment described here analyzed a MAT  $\alpha$  strain in which *MFA1* and *MFA2*, the genes encoding the  $\alpha$ -factor mating pheromone precursor, are normally repressed. In the isogenic *tup1* $\Delta$  strain, these genes were inappropriately expressed, reflecting the role that Tup1 plays in their repression. Had we instead carried out this experiment with a MATA strain (in which expression of *MFA1* and *MFA2* is not repressed), it would not have been possible to conclude anything regarding the role of Tup1 in the repression of these genes. Conversely, we cannot distinguish indirect effects of the chronic absence of Tup1 in the mutant strain from effects directly attributable to its participation in repressing the transcription of a gene.

Another simple route to modulating the activity of a regulatory factor is to overexpress the gene that encodes it. *YAP1* encodes a DNA-binding transcription factor belonging to the b-zip class of DNA-binding proteins. Overexpression of *YAP1* in yeast confers increased resistance to hydrogen peroxide, *o*-phenanthroline, heavy metals, and osmotic stress (45). We analyzed differential gene expression between a wild-type strain bearing a control plasmid and a strain with a plasmid expressing *YAP1* under the control of the strong *GAL1-10* promoter, both grown in galactose (that is, a condition that induces *YAP1* overexpression). Complementary DNA from the control and *YAP1* overexpressing strains, labeled with Cy3 and Cy5, respectively, was prepared from mRNA isolated from the two strains and hybridized to the microarray. Thus, red spots on the array represent genes that were induced in the strain overexpressing *YAP1*.

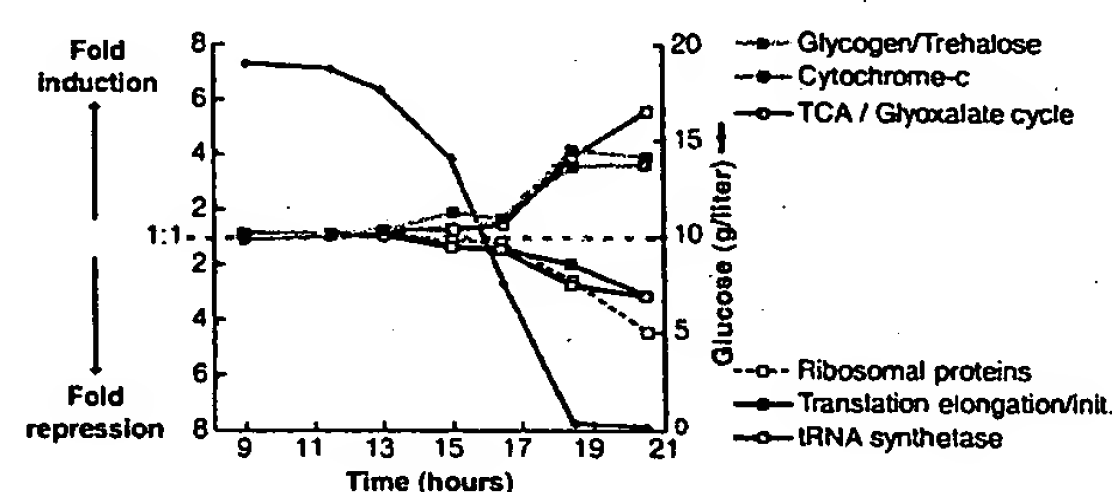
Of the 17 genes whose mRNA levels increased by more than threefold when

*YAP1* was overexpressed in this way, five bear homology to aryl-alcohol oxidoreductases (Fig. 2 and Table 1). An additional four of the genes in this set also belong to the general class of dehydrogenases/oxidoreductases. Very little is known about the role of aryl-alcohol oxidoreductases in *S. cerevisiae*, but these enzymes have been isolated from ligninolytic fungi, in which they participate in coupled redox reactions, oxidizing aromatic, and aliphatic unsaturated alcohols to aldehydes with the production of hydrogen peroxide (46, 47). The fact that a remarkable fraction of the targets identified in this experiment belong to the same small, functional group of oxidoreductases suggests that these genes

might play an important protective role during oxidative stress. Transcription of a small number of genes was reduced in the strain overexpressing Yap1. Interestingly, many of these genes encode sugar permeases or enzymes involved in inositol metabolism.

We searched for Yap1-binding sites (TTACTAA or TGAATA) in the sequences upstream of the target genes we identified (48). About two-thirds of the genes that were induced by more than threefold upon Yap1 overexpression had one or more binding sites within 600 bases upstream of the start codon (Table 1), suggesting that they are directly regulated by Yap1. The absence of canonical Yap1-bind-

**Fig. 4.** Coordinated regulation of functionally related genes. The curves represent the average induction or repression ratios for all the genes in each indicated group. The total number of genes in each group was as follows: ribosomal proteins, 112; translation elongation and initiation factors, 25; tRNA synthetases (excluding mitochondrial synthetases), 17; glycogen and trehalose synthesis and degradation, 15; cytochrome c oxidase and reductase proteins, 19; and TCA- and glyoxylate-cycle enzymes, 24.



**Table 1.** Genes induced by *YAP1* overexpression. This list includes all the genes for which mRNA levels increased by more than twofold upon *YAP1* overexpression in both of two duplicate experiments, and for which the average increase in mRNA level in the two experiments was greater than threefold (50). Positions of the canonical Yap1 binding sites upstream of the start codon, when present, and the average fold-increase in mRNA levels measured in the two experiments are indicated.

ORF	Distance of Yap1 site from ATG	Gene	Description	Fold-increase
YNL331C			Putative aryl-alcohol reductase	12.9
YKL071W			Similarity to bacterial csgA protein	10.4
YML007W	162-222 (5 sites)	YAP1	Transcriptional activator involved in oxidative stress response	9.8
YFL056C	223, 242		Homology to aryl-alcohol dehydrogenases	9.0
YLL060C	98		Putative glutathione transferase	7.4
YOL165C	266		Putative aryl-alcohol dehydrogenase (NADP+)	7.0
YCR107W			Putative aryl-alcohol reductase	6.5
YML116W	409	ATR1	Aminotriazole and 4-nitroquinoline resistance protein	6.5
YBR008C	142, 167, 364		Homology to benomyl/methotrexate resistance protein	6.1
YCLX08C			Hypothetical protein	6.1
YJR155W			Putative aryl-alcohol dehydrogenase	6.0
YPL171C	148, 212	OYE3	NAPDH dehydrogenase (old yellow enzyme), isoform 3	5.8
YLR460C	167, 317		Homology to hypothetical proteins YCR102c and YNL134c	4.7
YKR076W	178		Homology to hypothetical protein YMR251w	4.5
YHR179W	327	OYE2	NAD(P)H oxidoreductase (old yellow enzyme), isoform 1	4.1
YML131W	507		Similarity to <i>A. thaliana</i> zeta-crystallin homolog	3.7
YOL126C		MDH2	Malate dehydrogenase	3.3

ing sites upstream of the others may reflect an ability of Yap1 to bind sites that differ from the canonical binding sites, perhaps in cooperation with other factors, or less likely, may represent an indirect effect of Yap1 overexpression, mediated by one or more intermediary factors. Yap1 sites were found only four times in the corresponding region of an arbitrary set of 30 genes that were not differentially regulated by Yap1.

Use of a DNA microarray to characterize the transcriptional consequences of mutations affecting the activity of regulatory molecules provides a simple and powerful approach to dissection and characterization of regulatory pathways and net-

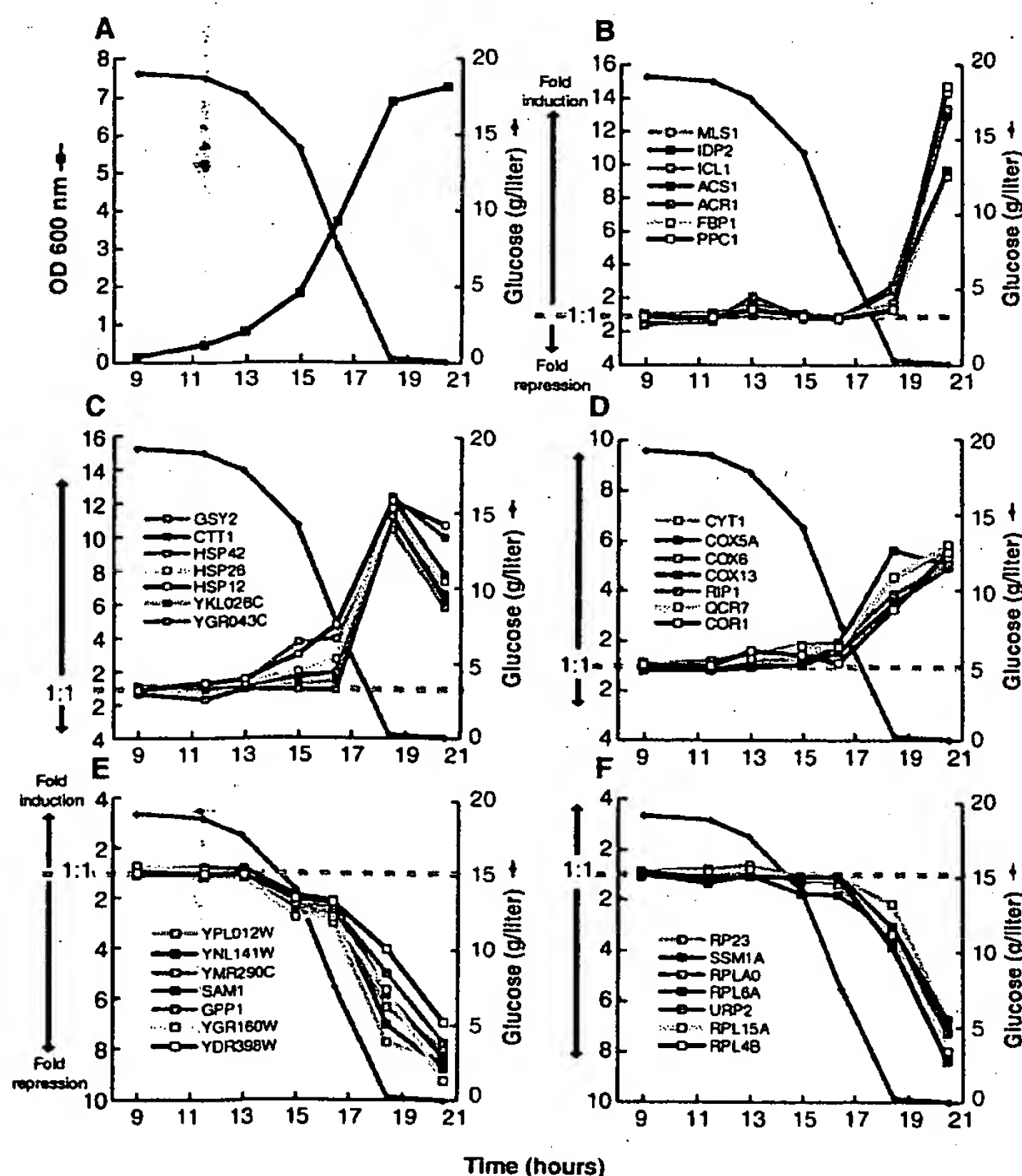
works. This strategy also has an important practical application in drug screening. Mutations in specific genes encoding candidate drug targets can serve as surrogates for the ideal chemical inhibitor or modulator of their activity. DNA microarrays can be used to define the resulting signature pattern of alterations in gene expression, and then subsequently used in an assay to screen for compounds that reproduce the desired signature pattern.

DNA microarrays provide a simple and economical way to explore gene expression patterns on a genomic scale. The hurdles to extending this approach to any other organism are minor. The equipment

required for fabricating and using DNA microarrays (9) consists of components that were chosen for their modest cost and simplicity. It was feasible for a small group to accomplish the amplification of more than 6000 genes in about 4 months and, once the amplified gene sequences were in hand, only 2 days were required to print a set of 110 microarrays of 6400 elements each. Probe preparation, hybridization, and fluorescent imaging are also simple procedures. Even conceptually simple experiments, as we described here, can yield vast amounts of information. The value of the information from each experiment of this kind will progressively increase as more is learned about the functions of each gene and as additional experiments define the global changes in gene expression in diverse other natural processes and genetic perturbations. Perhaps the greatest challenge now is to develop efficient methods for organizing, distributing, interpreting, and extracting insights from the large volumes of data these experiments will provide.

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8. Primers for each known or predicted protein coding sequence were supplied by Research Genetics. PCR was performed with the protocol supplied by Research Genetics, using genomic DNA from yeast strain S288C as a template. Each PCR product was verified by agarose gel electrophoresis and was deemed correct if the lane contained a single band of appropriate mobility. Failures were marked as such in the database. The overall success rate for a single-pass amplification of 6116 ORFs was ~94.5%.
9. Glass slides (Gold Seal) were cleaned for 2 hours in a solution of 2 N NaOH and 70% ethanol. After rinsing in distilled water, the slides were then treated with a 1:5 dilution of poly-L-lysine adhesive solution (Sigma) for 1 hour, and then dried for 5 min at 40°C in a vacuum oven. DNA samples from 100- $\mu$ l PCR reactions were purified by ethanol purification in 96-well microtiter plates. The resulting precipitates were resuspended in 3 $\times$  standard saline citrate (SSC) and transferred to new plates for arraying. A custom-built arraying robot was used to print on a batch of 110 slides. Details of the design of the microarrayer are available at [cmgm.stanford.edu/pbrown](http://cmgm.stanford.edu/pbrown). After printing, the microarrays were rehydrated for 30 s in a humid chamber and then snap-dried for 2 s on a hot plate (100°C). The DNA was then ultraviolet (UV)-crosslinked to the surface by subjecting the slides to 60 mJ of energy (Stratagene Stratalinker). The rest of the poly-L-lysine surface was blocked by a 15-min incubation in a solution of 70 mM succinic anhydride dissolved in a solution consisting of 315 ml of 1-methyl-2-pyrrolidinone (Aldrich) and 35 ml of 1 M boric acid (pH 8.0). Directly after the blocking reac-



**Fig. 5.** Distinct temporal patterns of induction or repression help to group genes that share regulatory properties. (A) Temporal profile of the cell density, as measured by OD at 600 nm and glucose concentration in the media. (B) Seven genes exhibited a strong induction (greater than ninefold) only at the last timepoint (20.5 hours). With the exception of *IDP2*, each of these genes has a CSRE UAS. There were no additional genes observed to match this profile. (C) Seven members of a class of genes marked by early induction with a peak in mRNA levels at 18.5 hours. Each of these genes contains STRE motif repeats in their upstream promoter regions. (D) Cytochrome c oxidase and ubiquinol cytochrome c reductase genes. Marked by an induction coincident with the diauxic shift, each of these genes contains a consensus binding motif for the HAP2,3,4 protein complex. At least 17 genes shared a similar expression profile. (E) *SAM1*, *GPP1*, and several genes of unknown function are repressed before the diauxic shift, and continue to be repressed upon entry into stationary phase. (F) Ribosomal protein genes comprise a large class of genes that are repressed upon depletion of glucose. Each of the genes profiled here contains one or more RAP1-binding motifs upstream of its promoter. RAP1 is a transcriptional regulator of most ribosomal proteins.



- tion, the bound DNA was denatured by a 2-min incubation in distilled water at  $-95^{\circ}\text{C}$ . The slides were then transferred into a bath of 100% ethanol at room temperature, rinsed, and then spun dry in a clinical centrifuge. Slides were stored in a closed box at room temperature until used.
10. YPD medium (8 liters), in a 10-liter fermentation vessel, was inoculated with 2 ml of a fresh overnight culture of yeast strain DBY7286 (MATa, ura3, GAL2). The fermentor was maintained at  $30^{\circ}\text{C}$  with constant agitation and aeration. The glucose content of the media was measured with a UV test kit (Boehringer Mannheim, catalog number 716251). Cell density was measured by OD at 600-nm wavelength. Aliquots of culture were rapidly withdrawn from the fermentation vessel by peristaltic pump, spun down at room temperature, and then flash frozen with liquid nitrogen. Frozen cells were stored at  $-80^{\circ}\text{C}$ .
  11. Cy3-dUTP or Cy5-dUTP (Amersham) was incorporated during reverse transcription of 1.25  $\mu\text{g}$  of polyadenylated [poly(A)<sup>+</sup>] RNA, primed by a dT(16) oligomer. This mixture was heated to  $70^{\circ}\text{C}$  for 10 min, and then transferred to ice. A premixed solution, consisting of 200 U Superscript II (Gibco), buffer, deoxyribonucleoside triphosphates, and fluorescent nucleotides, was added to the RNA. Nucleotides were used at these final concentrations: 500  $\mu\text{M}$  for dATP, dCTP, and dGTP and 200  $\mu\text{M}$  for dTTP. Cy3-dUTP and Cy5-dUTP were used at a final concentration of 100  $\mu\text{M}$ . The reaction was then incubated at  $42^{\circ}\text{C}$  for 2 hours. Unincorporated fluorescent nucleotides were removed by first diluting the reaction mixture with 470  $\mu\text{l}$  of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA and then subsequently concentrating the mix to  $\sim 5 \mu\text{l}$ , using Centricon-30 microconcentrators (Amicon).
  12. Purified, labeled cDNA was resuspended in 11  $\mu\text{l}$  of  $3.5\times$  SSC containing 10  $\mu\text{g}$  poly(dA) and 0.3  $\mu\text{l}$  of 10% SDS. Before hybridization, the solution was boiled for 2 min and then allowed to cool to room temperature. The solution was applied to the microarray under a cover slip, and the slide was placed in a custom hybridization chamber which was subsequently incubated for  $\sim 8$  to 12 hours in a water bath at  $62^{\circ}\text{C}$ . Before scanning, slides were washed in  $2\times$  SSC, 0.2% SDS for 5 min, and then  $0.05\times$  SSC for 1 min. Slides were dried before scanning by centrifugation at 500 rpm in a Beckman CS-6R centrifuge.
  13. The complete data set is available on the Internet at [cmgm.stanford.edu/pbrown/explore/index.html](http://cmgm.stanford.edu/pbrown/explore/index.html)
  14. For 95% of all the genes analyzed, the mRNA levels measured in cells harvested at the first and second interval after inoculation differed by a factor of less than 1.5. The correlation coefficient for the comparison between mRNA levels measured for each gene in these two different mRNA samples was 0.98. When duplicate mRNA preparations from the same cell sample were compared in the same way, the correlation coefficient between the expression levels measured for the two samples by comparative hybridization was 0.99.
  15. The numbers and identities of known and putative genes, and their homologies to other genes, were gathered from the following public databases: *Saccharomyces* Genome Database ([genome-www.stanford.edu](http://genome-www.stanford.edu)), Yeast Protein Database ([quest7.proteome.com](http://quest7.proteome.com)), and Munich Information Centre for Protein Sequences ([speedy.mips.biochem.mpg.de/mips/yeast/index.htm](http://speedy.mips.biochem.mpg.de/mips/yeast/index.htm)).
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  29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide codes are as follows: B-C, G, or T; N-G, A, T, or C; R-A or G; and Y-C or T.
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  36. For example, we observed large inductions of the genes coding for *PCK1*, *FBP1* [Z. Yin et al., *Mol. Microbiol.* 20, 751 (1996)], the central glyoxylate cycle gene *ICL1* [A. Scholer and H. J. Schuller, *Curr. Genet.* 23, 375 (1993)], and the "aerobic" isoform of acetyl-CoA synthase, *ACS1* [M. A. van den Berg et al., *J. Biol. Chem.* 271, 28953 (1996)], with concomitant down-regulation of the glycolytic-specific genes *PYK1* and *PFK2* [P. A. Moore et al., *Mol. Cell. Biol.* 11, 5330 (1991)]. Other genes not directly involved in carbon metabolism but known to be induced upon nutrient limitation include genes encoding cytosolic catalase *CTT1* [P. H. Bissinger et al., *ibid.* 9, 1309 (1989)] and several genes encoding small heat-shock proteins, such as *HSP12*, *HSP26*, and *HSP42* [I. Farkas et al., *J. Biol. Chem.* 266, 15602 (1991); U. M. Praekelt and P. A. Meacock, *Mol. Gen. Genet.* 223, 97 (1990); D. Wotton et al., *J. Biol. Chem.* 271, 2717 (1996)].
  37. The levels of induction we measured for genes that were expressed at very low levels in the uninduced state (notably, *FBP1* and *PCK1*) were generally lower than those previously reported. This discrepancy was likely due to the conservative background subtraction method we used, which generally resulted in overestimation of very low expression levels (46).
  38. Cross-hybridization of highly related sequences can also occasionally obscure changes in gene expression, an important concern where members of gene families are functionally specialized and differentially regulated. The major alcohol dehydrogenase genes, *ADH1* and *ADH2*, share 88% nucleotide identity. Reciprocal regulation of these genes is an important feature of the diauxic shift, but was not observed in this experiment, presumably because of cross-hybridization of the fluorescent cDNAs representing these two genes. Nevertheless, we were able to detect differential expression of closely related isoforms of other enzymes, such as *HXK1/HXK2* (77% identical) [P. Herrero et al., *Yeast* 11, 137 (1995)], *MLS1/DAL7* (73% identical) (20), and *PGM1/PGM2* (72% identical) [D. Oh, J. E. Hopper, *Mol. Cell. Biol.* 10, 1415 (1990)], in accord with previous studies. Use in the microarray of deliberately selected DNA sequences corresponding to the most divergent segments of homologous genes, in lieu of the complete gene sequences, should relieve this problem in many cases.
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  42. The *tup1Δ* mutation consists of an insertion of the LEU2 coding sequence, including a stop codon, between the ATG of *TUP1* and an Eco RI site 124 base pairs before the stop codon of the *TUP1* gene.
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  49. Microarrays were scanned using a custom-built scanning laser microscope built by S. Smith with software written by N. Ziv. Details concerning scanner design and construction are available at [cmgm.stanford.edu/pbrown](http://cmgm.stanford.edu/pbrown). Images were scanned at a resolution of 20  $\mu\text{m}$  per pixel. A separate scan, using the appropriate excitation line, was done for each of the two fluorophores used. During the scanning process, the ratio between the signals in the two channels was calculated for several array elements containing total genomic DNA. To normalize the two channels with respect to overall intensity, we then adjusted photomultiplier and laser power settings such that the signal ratio at these elements was as close to 1.0 as possible. The combined images were analyzed with custom-written software. A bounding box, fitted to the size of the DNA spots in each quadrant, was placed over each array element. The average fluorescent intensity was calculated by summing the intensities of each pixel present in a bounding box, and then dividing by the total number of pixels. Local area background was calculated for each array element by determining the average fluorescent intensity for the lower 20% of pixel intensities. Although this method tends to underestimate the background, causing an underestimation of extreme ratios, it produces a very consistent and noise-tolerant approximation. Although the analog-to-digital board used for data collection possesses a wide dynamic range (12 bits), several signals were saturated (greater than the maximum signal intensity allowed) at the chosen settings. Therefore, extreme ratios at bright elements are generally underestimated. A signal was deemed significant if the average intensity after background subtraction was at least 2.5-fold higher than the standard deviation in the background measurements for all elements on the array.
  50. In addition to the 17 genes shown in Table 1, three additional genes were induced by an average of more than threefold in the duplicate experiments, but in one of the two experiments, the induction was less than twofold (range 1.6- to 1.9-fold).
  51. We thank H. Bennett, P. Spellman, J. Ravetto, M. Eisen, R. Pillai, B. Dunn, T. Ferea, and other members of the Brown lab for their assistance and helpful advice. We also thank S. Friend, D. Botstein, S. Smith, J. Hudson, and D. Dolginow for advice, support, and encouragement; K. Struhl and S. Chatterjee for the *Tup1* deletion strain; L. Fernandes for helpful advice on Yap1; and S. Klapholz and the reviewers for many helpful comments on the manuscript. Supported by a grant from the National Human Genome Research Institute (NHGRI) (HG00450), and by the Howard Hughes Medical Institute (HHMI). J.D.R. was supported by the HHMI and the NHGRI. V.R. was supported in part by an Institutional Training Grant in Genome Science (T32 HG00044) from the NHGRI. P.O.B. is an associate investigator of the HHMI.

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# Assessing sequence comparison methods with reliable structurally identified distant evolutionary relationships

STEVEN E. BRENNER\*†‡, CYRUS CHOTHIA\*, AND TIM J. P. HUBBARD§

\*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom; and §Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambs CB10 1SA, United Kingdom

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**ABSTRACT** Pairwise sequence comparison methods have been assessed using proteins whose relationships are known reliably from their structures and functions, as described in the SCOP database [Murzin, A. G., Brenner, S. E., Hubbard, T. & Chothia C. (1995) *J. Mol. Biol.* 247, 536–540]. The evaluation tested the programs BLAST [Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410], WU-BLAST2 [Altschul, S. F. & Gish, W. (1996) *Methods Enzymol.* 266, 460–480], FASTA [Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2444–2448], and SSEARCH [Smith, T. F. & Waterman, M. S. (1981) *J. Mol. Biol.* 147, 195–197] and their scoring schemes. The error rate of all algorithms is greatly reduced by using statistical scores to evaluate matches rather than percentage identity or raw scores. The E-value statistical scores of SSEARCH and FASTA are reliable: the number of false positives found in our tests agrees well with the scores reported. However, the P-values reported by BLAST and WU-BLAST2 exaggerate significance by orders of magnitude. SSEARCH, FASTA ktup = 1, and WU-BLAST2 perform best, and they are capable of detecting almost all relationships between proteins whose sequence identities are >30%. For more distantly related proteins, they do much less well; only one-half of the relationships between proteins with 20–30% identity are found. Because many homologs have low sequence similarity, most distant relationships cannot be detected by any pairwise comparison method; however, those which are identified may be used with confidence.

Sequence database searching plays a role in virtually every branch of molecular biology and is crucial for interpreting the sequences issuing forth from genome projects. Given the method's central role, it is surprising that overall and relative capabilities of different procedures are largely unknown. It is difficult to verify algorithms on sample data because this requires large data sets of proteins whose evolutionary relationships are known unambiguously and independently of the methods being evaluated. However, nearly all known homologs have been identified by sequence analysis (the method to be tested). Also, it is generally very difficult to know, in the absence of structural data, whether two proteins that lack clear sequence similarity are unrelated. This has meant that although previous evaluations have helped improve sequence comparison, they have suffered from insufficient, imperfectly characterized, or artificial test data. Assessment also has been problematic because high quality database sequence searching attempts to have both sensitivity (detection of homologs) and specificity (rejection of unrelated proteins); however, these complementary goals are linked such that increasing one causes the other to be reduced.

Sequence comparison methodologies have evolved rapidly, so no previously published tests have evaluated modern versions of programs commonly used. For example, parameters in BLAST (1) have changed, and WU-BLAST2 (2)—which produces gapped alignments—has become available. The latest version of FASTA (3) previously tested was 1.6, but the current release (version 3.0) provides fundamentally different results in the form of statistical scoring.

The previous reports also have left gaps in our knowledge. For example, there has been no published assessment of thresholds for scoring schemes more sophisticated than percentage identity. Thus, the widely discussed statistical scoring measures have never actually been evaluated on large databases of real proteins. Moreover, the different scoring schemes commonly in use have not been compared.

Beyond these issues, there is a more fundamental question: in an absolute sense, how well does pairwise sequence comparison work? That is, what fraction of homologous proteins can be detected using modern database searching methods?

In this work, we attempt to answer these questions and to overcome both of the fundamental difficulties that have hindered assessment of sequence comparison methodologies. First, we use the set of distant evolutionary relationships in the SCOP: Structural Classification of Proteins database (4), which is derived from structural and functional characteristics (5). The SCOP database provides a uniquely reliable set of homologs, which are known independently of sequence comparison. Second, we use an assessment method that jointly measures both sensitivity and specificity. This method allows straightforward comparison of different sequence searching procedures. Further, it can be used to aid interpretation of real database searches and thus provide optimal and reliable results.

**Previous Assessments of Sequence Comparison.** Several previous studies have examined the relative performance of different sequence comparison methods. The most encompassing analyses have been by Pearson (6, 7), who compared the three most commonly used programs. Of these, the Smith–Waterman algorithm (8) implemented in SSEARCH (3) is the oldest and slowest but the most rigorous. Modern heuristics have provided BLAST (1) the speed and convenience to make it the most popular program. Intermediate between these two is FASTA (3), which may be run in two modes offering either greater speed (ktup = 2) or greater effectiveness (ktup = 1). Pearson also considered different parameters for each of these programs.

To test the methods, Pearson selected two representative proteins from each of 67 protein superfamilies defined by the PIR database (9). Each was used as a query to search the database, and the matched proteins were marked as being homologous or unrelated according to their membership of PIR

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Abbreviation: EPQ, errors per query.

†Present address: Department of Structural Biology, Stanford University, Fairchild Building D-109, Stanford, CA 94305-5126

‡To whom reprints requests should be addressed. e-mail: [brenner@hyper.stanford.edu](mailto:brenner@hyper.stanford.edu).



superfamilies. Pearson found that modern matrices and "ln-scaling" of raw scores improve results considerably. He also reported that the rigorous Smith-Waterman algorithm worked slightly better than FASTA, which was in turn more effective than BLAST.

Very large scale analyses of matrices have been performed (10), and Henikoff and Henikoff (11) also evaluated the effectiveness of BLAST and FASTA. Their test with BLAST considered the ability to detect homologs above a predetermined score but had no penalty for methods which also reported large numbers of spurious matches. The Henikoffs searched the SWISS-PROT database (12) and used PROSITE (13) to define homologous families. Their results showed that the BLOSUM62 matrix (14) performed markedly better than the extrapolated PAM-series matrices (15), which previously had been popular.

A crucial aspect of any assessment is the data that are used to test the ability of the program to find homologs. But in Pearson's and the Henikoffs' evaluations of sequence comparison, the correct results were effectively unknown. This is because the superfamilies in PIR and PROSITE are principally created by using the same sequence comparison methods which are being evaluated. Interdependency of data and methods creates a "chicken and egg" problem, and means for example, that new methods would be penalized for correctly identifying homologs missed by older programs. For instance, immunoglobulin variable and constant domains are clearly homologous, but PIR places them in different superfamilies. The problem is widespread: each superfamily in PIR 48.00 with a structural homolog is itself homologous to an average of 1.6 other PIR superfamilies (16).

To surmount these sorts of difficulties, Sander and Schneider (17) used protein structures to evaluate sequence comparison. Rather than comparing different sequence comparison algorithms, their work focused on determining a length-dependent threshold of percentage identity, above which all proteins would be of similar structure. A result of this analysis was the HSSP equation; it states that proteins with 25% identity over 80 residues will have similar structures, whereas shorter alignments require higher identity. (Other studies also have used structures (18–20), but these focused on a small number of model proteins and were principally oriented toward evaluating alignment accuracy rather than homology detection.)

A general solution to the problem of scoring comes from statistical measures (i.e., E-values and P-values) based on the extreme value distribution (21). Extreme value scoring was implemented analytically in the BLAST program using the Karlin and Altschul statistics (22, 23) and empirical approaches have been recently added to FASTA and SSEARCH. In addition to being heralded as a reliable means of recognizing significantly similar proteins (24, 25), the mathematical tractability of statistical scores "is a crucial feature of the BLAST algorithm" (1). The validity of this scoring procedure has been tested analytically and empirically (see ref. 2 and references in ref. 24). However, all large empirical tests used random sequences that may lack the subtle structure found within biological sequences (26, 27) and obviously do not contain any real homologs. Thus, although many researchers have suggested that statistical scores be used to rank matches (24, 25, 28), there have been no large rigorous experiments on biological data to determine the degree to which such rankings are superior.

**A Database for Testing Homology Detection.** Since the discovery that the structures of hemoglobin and myoglobin are very similar though their sequences are not (29), it has been apparent that comparing structures is a more powerful (if less convenient) way to recognize distant evolutionary relationships than comparing sequences. If two proteins show a high degree of similarity in their structural details and function, it

is very probable that they have an evolutionary relationship though their sequence similarity may be low.

The recent growth of protein structure information combined with the comprehensive evolutionary classification in the SCOP database (4, 5) have allowed us to overcome previous limitations. With these data, we can evaluate the performance of sequence comparison methods on real protein sequences whose relationships are known confidently. The SCOP database uses structural information to recognize distant homologs, the large majority of which can be determined unambiguously. These superfamilies, such as the globins or the immunoglobulins, would be recognized as related by the vast majority of the biological community despite the lack of high sequence similarity.

From SCOP, we extracted the sequences of domains of proteins in the Protein Data Bank (PDB) (30) and created two databases. One (PDB90D-B) has domains, which were all <90% identical to any other, whereas (PDB40D-B) had those <40% identical. The databases were created by first sorting all protein domains in SCOP by their quality and making a list. The highest quality domain was selected for inclusion in the database and removed from the list. Also removed from the list (and discarded) were all other domains above the threshold level of identity to the selected domain. This process was repeated until the list was empty. The PDB40D-B database contains 1,323 domains, which have 9,044 ordered pairs of distant relationships, or  $\approx 0.5\%$  of the total 1,749,006 ordered pairs. In PDB90D-B, the 2,079 domains have 53,988 relationships, representing 1.2% of all pairs. Low complexity regions of sequence can achieve spurious high scores, so these were masked in both databases by processing with the SEG program (27) using recommended parameters: 12 1.8 2.0. The databases used in this paper are available from <http://sss.stanford.edu/sss/>, and databases derived from the current version of SCOP may be found at <http://scop.mrc-lmb.cam.ac.uk/scop/>.

Analyses from both databases were generally consistent, but PDB40D-B focuses on distantly related proteins and reduces the heavy overrepresentation in the PDB of a small number of families (31, 32), whereas PDB90D-B (with more sequences) improves evaluations of statistics. Except where noted otherwise, the distant homolog results here are from PDB40D-B. Although the precise numbers reported here are specific to the structural domain databases used, we expect the trends to be general.

**Assessment Data and Procedure.** Our assessment of sequence comparison may be divided into four different major categories of tests. First, using just a single sequence comparison algorithm at a time, we evaluated the effectiveness of different scoring schemes. Second, we assessed the reliability of scoring procedures, including an evaluation of the validity of statistical scoring. Third, we compared sequence comparison algorithms (using the optimal scoring scheme) to determine their relative performance. Fourth, we examined the distribution of homologs and considered the power of pairwise sequence comparison to recognize them. All of the analyses used the databases of structurally identified homologs and a new assessment criterion.

The analyses tested BLAST (1), version 1.4.9MP, and WU-BLAST2 (2), version 2.0a13MP. Also assessed was the FASTA package, version 3.0t76 (3), which provided FASTA and the SSEARCH implementation of Smith-Waterman (8). For SSEARCH and FASTA, we used BLOSUM45 with gap penalties  $-12/-1$  (7, 16). The default parameters and matrix (BLOSUM62) were used for BLAST and WU-BLAST2.

**The "Coverage Vs. Error" Plot.** To test a particular protocol (comprising a program and scoring scheme), each sequence from the database was used as a query to search the database. This yielded ordered pairs of query and target sequences with associated scores, which were sorted, on the basis of their scores, from best to worst. The ideal method would have



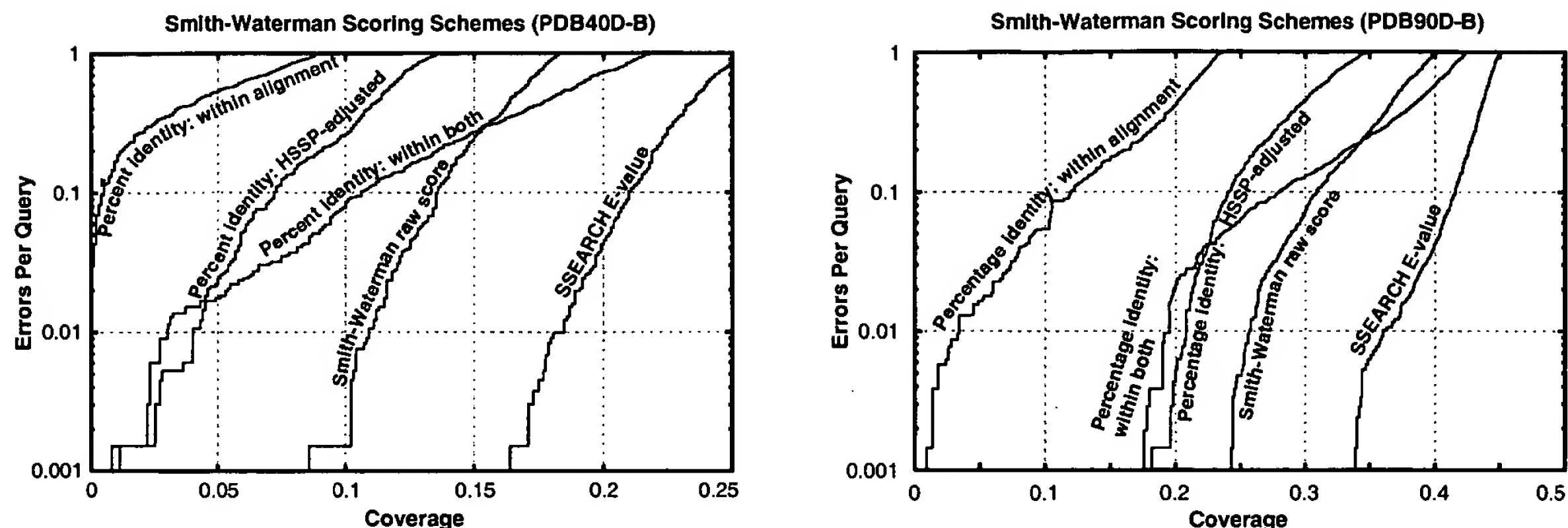


FIG. 1. Coverage vs. error plots of different scoring schemes for SSEARCH Smith-Waterman. (A) Analysis of PDB40D-B database. (B) Analysis of PDB90D-B database. All of the proteins in the database were compared with each other using the SSEARCH program. The results of this single set of comparisons were considered using five different scoring schemes and assessed. The graphs show the coverage and errors per query (EPQ) for statistical scores, raw scores, and three measures using percentage identity. In the coverage vs. error plot, the  $x$  axis indicates the fraction of all homologs in the database (known from structure) which have been detected. Precisely, it is the number of detected pairs of proteins with the same fold divided by the total number of pairs from a common superfamily. PDB40D-B contains a total of 9,044 homologs, so a score of 10% indicates identification of 904 relationships. The  $y$  axis reports the number of EPQ. Because there are 1,323 queries made in the PDB40D-B all-vs.-all comparison, 13 errors corresponds to 0.01, or 1% EPQ. The  $y$  axis is presented on a log scale to show results over the widely varying degrees of accuracy which may be desired. The scores that correspond to the levels of EPQ and coverage are shown in Fig. 4 and Table 1. The graph demonstrates the trade-off between sensitivity and selectivity. As more homologs are found (moving to the right), more errors are made (moving up). The ideal method would be in the lower right corner of the graph, which corresponds to identifying many evolutionary relationships without selecting unrelated proteins. Three measures of percentage identity are plotted. Percentage identity within alignment is the degree of identity within the aligned region of the proteins, without consideration of the alignment length. Percentage identity within both is the number of identical residues in the aligned region as a percentage of the average length of the query and target proteins. The HSSP equation (17) is  $H = 290.15l^{-0.562}$  where  $l$  is length for  $10 < l < 80$ ;  $H > 100$  for  $l < 10$ ;  $H = 24.7$  for  $l > 80$ . The percentage identity HSSP-adjusted score is the percent identity within the alignment minus  $H$ . Smith-Waterman raw scores and E-values were taken directly from the sequence comparison program.

perfect separation, with all of the homologs at the top of the list and unrelated proteins below. In practice, perfect separation is impossible to achieve so instead one is interested in drawing a threshold above which there are the largest number of related pairs of sequences consistent with an acceptable error rate.

Our procedure involved measuring the coverage and error for every threshold. Coverage was defined as the fraction of structurally determined homologs that have scores above the selected threshold; this reflects the sensitivity of a method. Errors per query (EPQ), an indicator of selectivity, is the number of nonhomologous pairs above the threshold divided by the number of queries. Graphs of these data, called coverage vs. error plots, were devised to understand how

protocols compare at different levels of accuracy. These graphs share effectively all of the beneficial features of Receiver Operating Characteristic (ROC) plots (33, 34) but better represent the high degrees of accuracy required in sequence comparison and the huge background of nonhomologs.

This assessment procedure is directly relevant to practical sequence database searching, for it provides precisely the information necessary to perform a reliable sequence database search. The EPQ measure places a premium on score consistency; that is, it requires scores to be comparable for different queries. Consistency is an aspect which has been largely

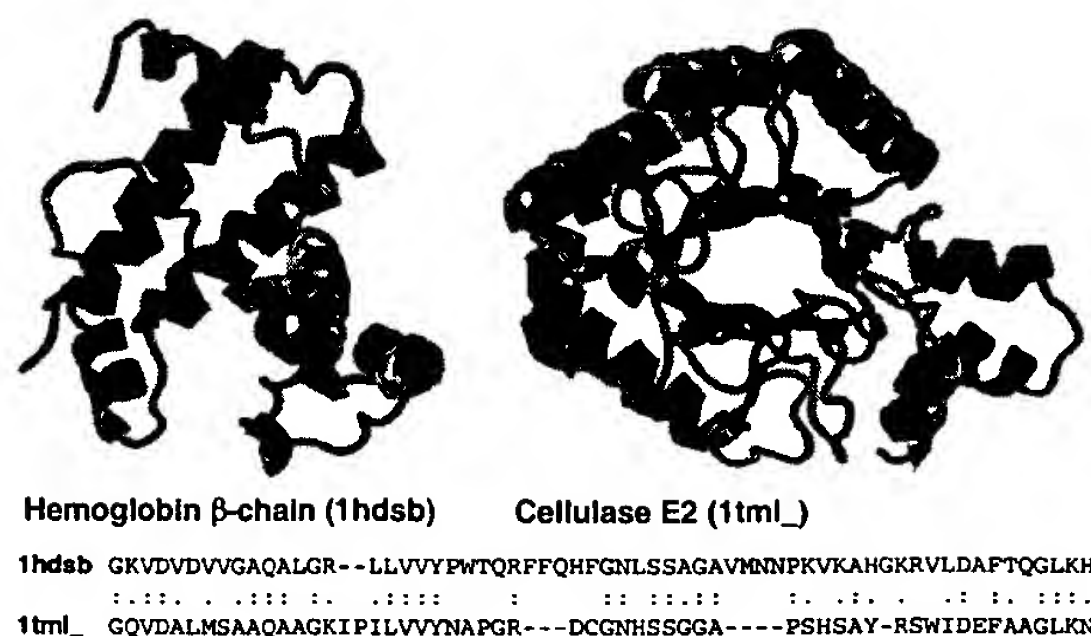


FIG. 2. Unrelated proteins with high percentage identity. Hemoglobin  $\beta$ -chain (PDB code 1hds chain b, ref. 38, *Left*) and cellulase E2 (PDB code 1tml, ref. 39, *Right*) have 39% identity over 64 residues, a level which is often believed to be indicative of homology. Despite this high degree of identity, their structures strongly suggest that these proteins are not related. Appropriately, neither the raw alignment score of 85 nor the E-value of 1.3 is significant. Proteins rendered by RASMOL (40).

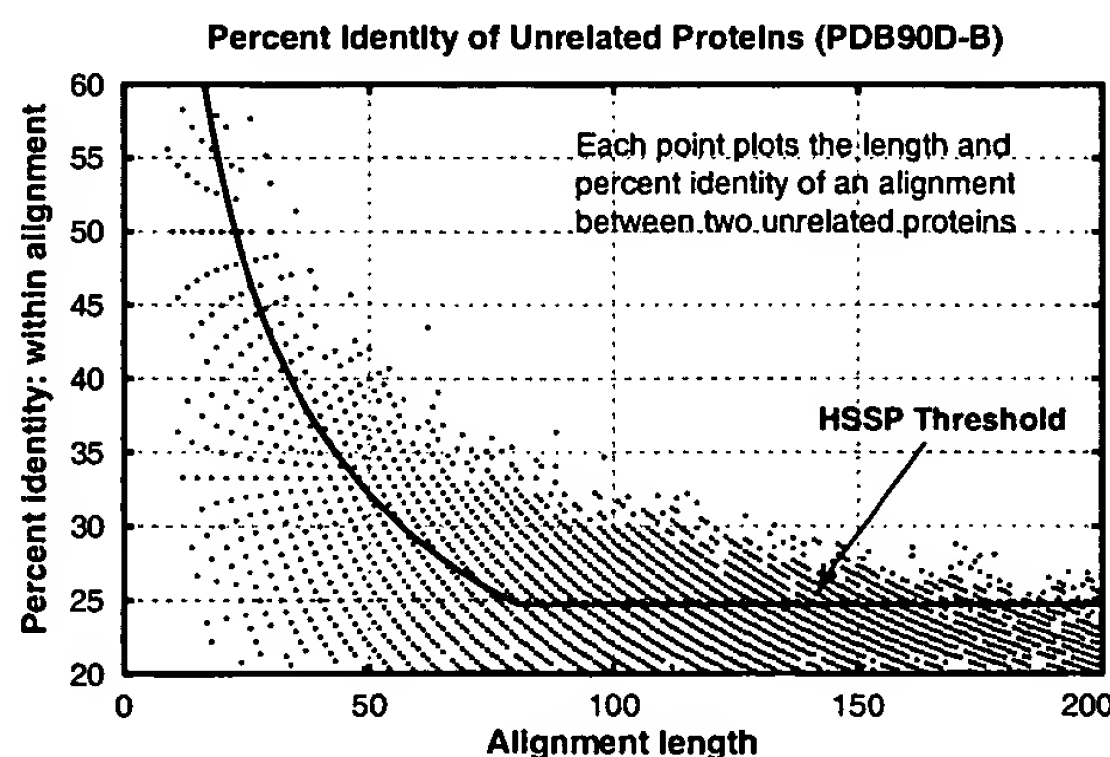


FIG. 3. Length and percentage identity of alignments of unrelated proteins in PDB90D-B: Each pair of nonhomologous proteins found with SSEARCH is plotted as a point whose position indicates the length and the percentage identity within the alignment. Because alignment length and percentage identity are quantized, many pairs of proteins may have exactly the same alignment length and percentage identity. The line shows the HSSP threshold (though it is intended to be applied with a different matrix and parameters).

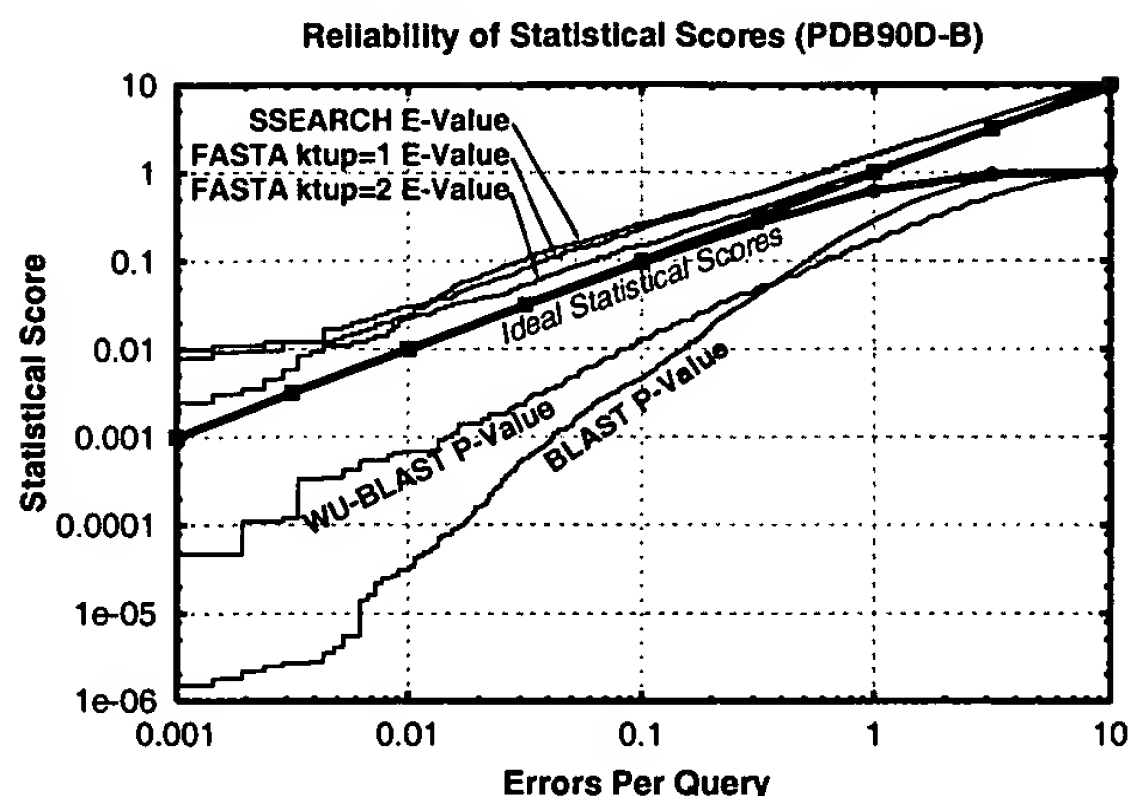


FIG. 4. Reliability of statistical scores in PDB90D-B: Each line shows the relationship between reported statistical score and actual error rate for a different program. E-values are reported for SSEARCH and FASTA, whereas P-values are shown for BLAST and WU-BLAST2. If the scoring were perfect, then the number of errors per query and the E-values would be the same, as indicated by the upper bold line. (P-values should be the same as EPQ for small numbers, and diverges at higher values, as indicated by the lower bold line.) E-values from SSEARCH and FASTA are shown to have good agreement with EPQ but underestimate the significance slightly. BLAST and WU-BLAST2 are overconfident, with the degree of exaggeration dependent upon the score. The results for PDB40D-B were similar to those for PDB90D-B despite the difference in number of homologs detected. This graph could be used to roughly calibrate the reliability of a given statistical score.

ignored in previous tests but is essential for the straightforward or automatic interpretation of sequence comparison results. Further, it provides a clear indication of the confidence that should be ascribed to each match. Indeed, the EPQ measure should approximate the expectation value reported by database searching programs, if the programs' estimates are accurate.

**The Performance of Scoring Schemes.** All of the programs tested could provide three fundamental types of scores. The first score is the percentage identity, which may be computed in several ways based on either the length of the alignment or the lengths of the sequences. The second is a "raw" or "Smith-Waterman" score, which is the measure optimized by the Smith-Waterman algorithm and is computed by summing the substitution matrix scores for each position in the alignment and subtracting gap penalties. In BLAST, a measure

related to this score is scaled into bits. Third is a statistical score based on the extreme value distribution. These results are summarized in Fig. 1.

**Sequence Identity.** Though it has been long established that percentage identity is a poor measure (35), there is a common rule-of-thumb stating that 30% identity signifies homology. Moreover, publications have indicated that 25% identity can be used as a threshold (17, 36). We find that these thresholds, originally derived years ago, are not supported by present results. As databases have grown, so have the possibilities for chance alignments with high identity; thus, the reported cutoffs lead to frequent errors. Fig. 2 shows one of the many pairs of proteins with very different structures that nonetheless have high levels of identity over considerable aligned regions. Despite the high identity, the raw and the statistical scores for such incorrect matches are typically not significant. The principal reasons percentage identity does so poorly seem to be that it ignores information about gaps and about the conservative or radical nature of residue substitutions.

From the PDB90D-B analysis in Fig. 3, we learn that 30% identity is a reliable threshold for this database only for sequence alignments of at least 150 residues. Because one unrelated pair of proteins has 43.5% identity over 62 residues, it is probably necessary for alignments to be at least 70 residues in length before 40% is a reasonable threshold, for a database of this particular size and composition.

At a given reliability, scores based on percentage identity detect just a fraction of the distant homologs found by statistical scoring. If one measures the percentage identity in the aligned regions without consideration of alignment length, then a negligible number of distant homologs are detected. Use of the HSP equation improves the value of percentage identity, but even this measure can find only 4% of all known homologs at 1% EPQ. In short, percentage identity discards most of the information measured in a sequence comparison.

**Raw Scores.** Smith-Waterman raw scores perform better than percentage identity (Fig. 1), but ln-scaling (7) provided no notable benefit in our analysis. It is necessary to be very precise when using either raw or bit scores because a 20% change in cutoff score could yield a tenfold difference in EPQ. However, it is difficult to choose appropriate thresholds because the reliability of a bit score depends on the lengths of the proteins matched and the size of the database. Raw score thresholds also are affected by matrix and gap parameters.

**Statistical Scores.** Statistical scores were introduced partly to overcome the problems that arise from raw scores. This scoring scheme provides the best discrimination between homologous proteins and those which are unrelated. Most

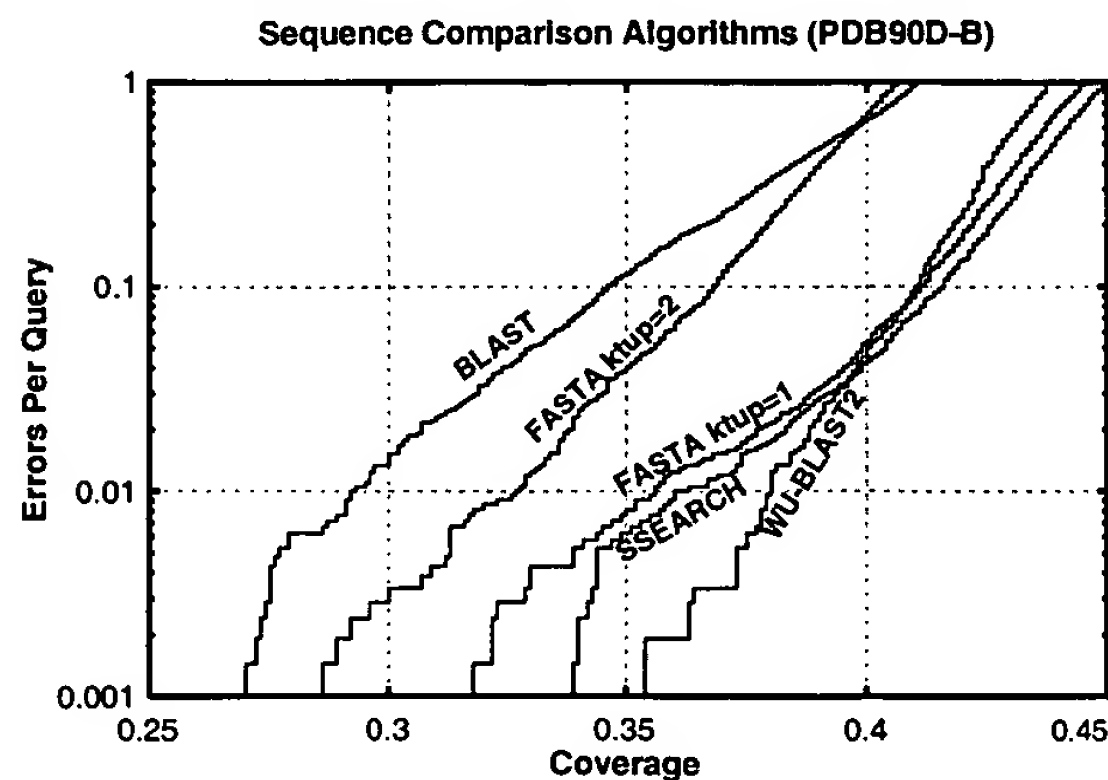
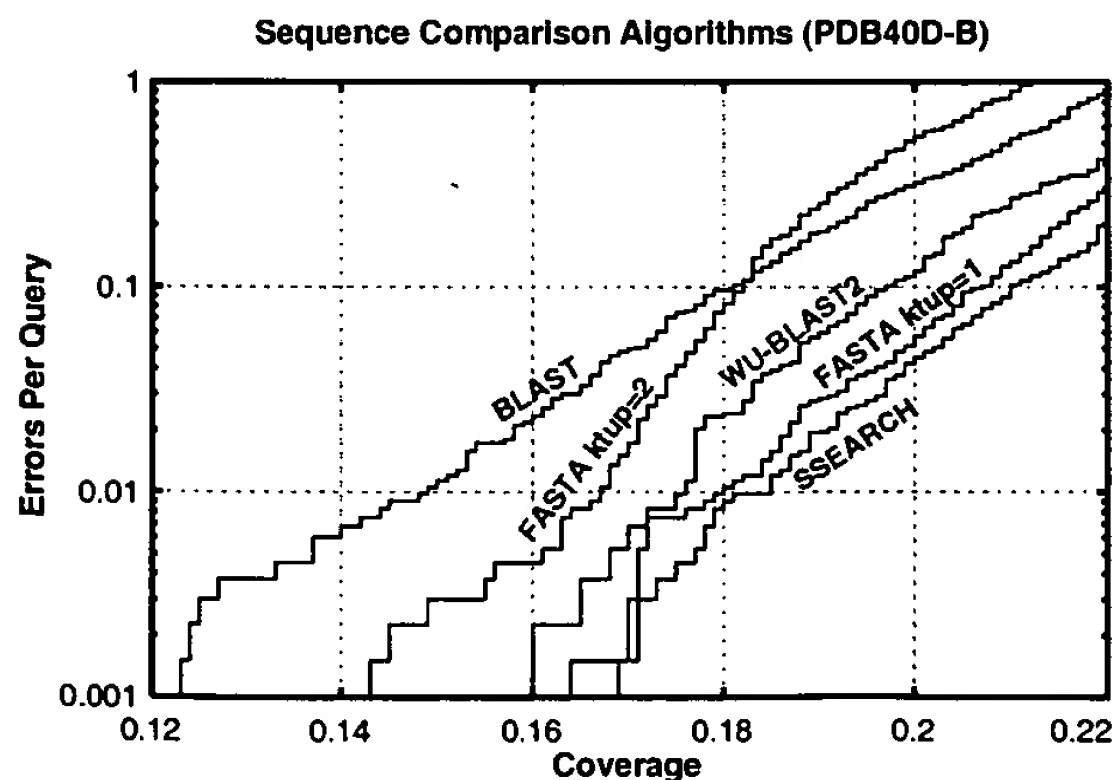


FIG. 5. Coverage vs. error plots of different sequence comparison methods: Five different sequence comparison methods are evaluated, each using statistical scores (E- or P-values). (A) PDB40D-B database. In this analysis, the best method is the slow SSEARCH, which finds 18% of relationships at 1% EPQ. FASTA ktup = 1 and WU-BLAST2 are almost as good. (B) PDB90D-B database. The quick WU-BLAST2 program provides the best coverage at 1% EPQ on this database, although at higher levels of error it becomes slightly worse than FASTA ktup = 1 and SSEARCH.

likely, its power can be attributed to its incorporation of more information than any other measure; it takes account of the full substitution and gap data (like raw scores) but also has details about the sequence lengths and composition and is scaled appropriately.

We find that statistical scores are not only powerful, but also easy to interpret. SSEARCH and FASTA show close agreement between statistical scores and actual number of errors per query (Fig. 4). The expectation value score gives a good, slightly conservative estimate of the chances of the two sequences being found at random in a given query. Thus, an E-value of 0.01 indicates that roughly one pair of nonhomologs of this similarity should be found in every 100 different queries. Neither raw scores nor percentage identity can be interpreted in this way, and these results validate the suitability of the extreme value distribution for describing the scores from a database search.

The P-values from BLAST also should be directly interpretable but were found to overstate significance by more than two orders of magnitude for 1% EPQ for this database. Nonetheless, these results strongly suggest that the analytic theory is fundamentally appropriate. WU-BLAST2 scores were more reliable than those from BLAST, but also exaggerate expected confidence by more than an order of magnitude at 1% EPQ.

**Overall Detection of Homologs and Comparison of Algorithms.** The results in Fig. 5A and Table 1 show that pairwise sequence comparison is capable of identifying only a small fraction of the homologous pairs of sequences in PDB40D-B. Even SSEARCH with E-values, the best protocol tested, could find only 18% of all relationships at a 1% EPQ. BLAST, which identifies 15%, was the worst performer, whereas FASTA  $k_{\text{tup}} = 1$  is nearly as effective as SSEARCH. FASTA  $k_{\text{tup}} = 2$  and WU-BLAST2 are intermediate in their ability to detect homologs. Comparison of different algorithms indicates that those capable of identifying more homologs are generally slower. SSEARCH is 25 times slower than BLAST and 6.5 times slower than FASTA  $k_{\text{tup}} = 1$ . WU-BLAST2 is slightly faster than FASTA  $k_{\text{tup}} = 2$ , but the latter has more interpretable scores.

In PDB90D-B, where there are many close relationships, the best method can identify only 38% of structurally known homologs (Fig. 5B). The method which finds that many relationships is WU-BLAST2. Consequently, we infer that the differences between FASTA  $k_{\text{tup}} = 1$ , SSEARCH, and WU-BLAST2 programs are unlikely to be significant when compared with variation in database composition and scoring reliability.

Fig. 6 helps to explain why most distant homologs cannot be found by sequence comparison: a great many such relationships have no more sequence identity than would be expected by chance. SSEARCH with E-values can recognize >90% of the homologous pairs with 30–40% identity. In this region, there are 30 pairs of homologous proteins that do not have significant E-values, but 26 of these involve sequences with <50 residues. Of sequences having 25–30% identity, 75% are identified by SSEARCH E-values. However, although the number of homologs grows at lower levels of identity, the detection falls off sharply: only 40% of homologs with 20–25% identity

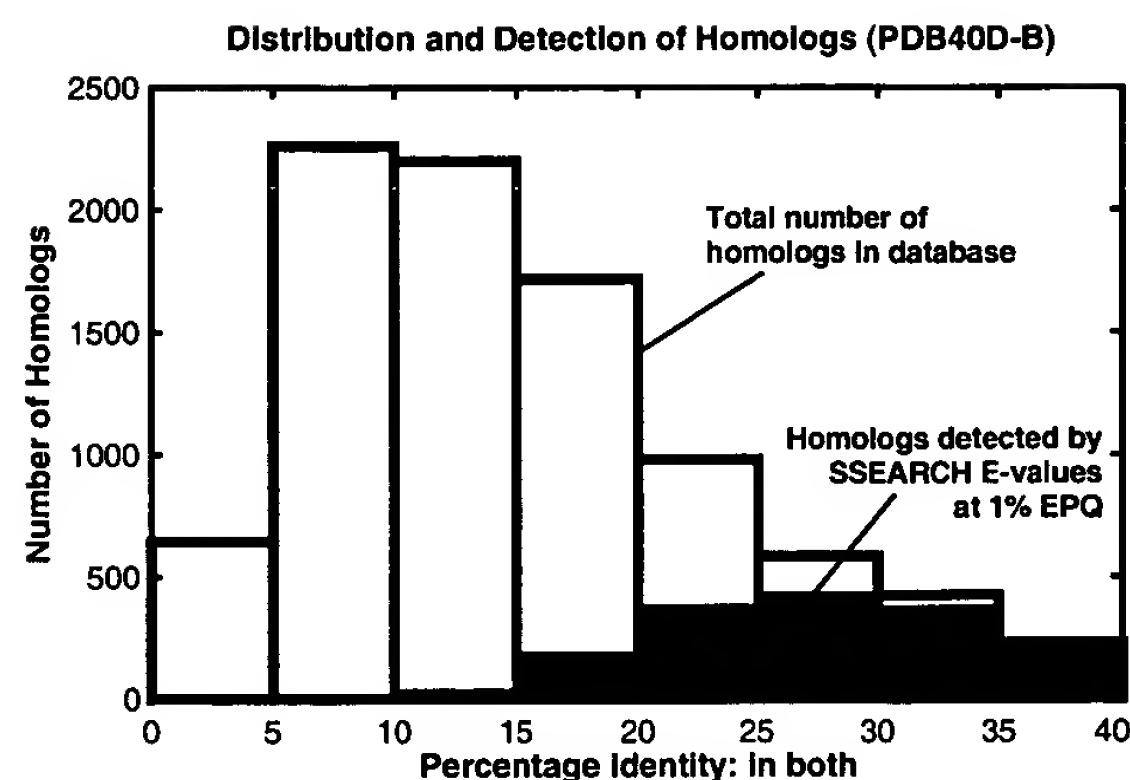


FIG. 6. Distribution and detection of homologs in PDB40D-B. Bars show the distribution of homologous pairs PDB40D-B according to their identity (using the measure of identity in both). Filled regions indicate the number of these pairs found by the best database searching method (SSEARCH with E-values) at 1% EPQ. The PDB40D-B database contains proteins with <40% identity, and as shown on this graph, most structurally identified homologs in the database have diverged extremely far in sequence and have <20% identity. Note that the alignments may be inaccurate, especially at low levels of identity. Filled regions show that SSEARCH can identify most relationships that have 25% or more identity, but its detection wanes sharply below 25%. Consequently, the great sequence divergence of most structurally identified evolutionary relationships effectively defeats the ability of pairwise sequence comparison to detect them.

are detected and only 10% of those with 15–20% can be found. These results show that statistical scores can find related proteins whose identity is remarkably low; however, the power of the method is restricted by the great divergence of many protein sequences.

After completion of this work, a new version of pairwise BLAST was released: BLASTGP (37). It supports gapped alignments, like WU-BLAST2, and dispenses with sum statistics. Our initial tests on BLASTGP using default parameters show that its E-values are reliable and that its overall detection of homologs was substantially better than that of ungapped BLAST, but not quite equal to that of WU-BLAST2.

## CONCLUSION

The general consensus amongst experts (see refs. 7, 24, 25, 27 and references therein) suggests that the most effective sequence searches are made by (i) using a large current database in which the protein sequences have been complexity masked and (ii) using statistical scores to interpret the results. Our experiments fully support this view.

Our results also suggest two further points. First, the E-values reported by FASTA and SSEARCH give fairly accurate estimates of the significance of each match, but the P-values provided by BLAST and WU-BLAST2 underestimate the true

Table 1. Summary of sequence comparison methods with PDB40D-B

Method	Relative Time*	1% EPQ Cutoff	Coverage at 1% EPQ
SSEARCH % identity: within alignment	25.5	>70%	<0.1
SSEARCH % identity: within both	25.5	34%	3.0
SSEARCH % identity: HSSP-scaled	25.5	35% (HSSP + 9.8)	4.0
SSEARCH Smith–Waterman raw scores	25.5	142	10.5
SSEARCH E-values	25.5	0.03	18.4
FASTA $k_{\text{tup}} = 1$ E-values	3.9	0.03	17.9
FASTA $k_{\text{tup}} = 2$ E-values	1.4	0.03	16.7
WU-BLAST2 P-values	1.1	0.003	17.5
BLAST P-values	1.0	0.00016	14.8

\*Times are from large database searches with genome proteins.



extent of errors. Second, SSEARCH, WU-BLAST2, and FASTA ktup = 1 perform best, though BLAST and FASTA ktup = 2 detect most of the relationships found by the best procedures and are appropriate for rapid initial searches.

The homologous proteins that are found by sequence comparison can be distinguished with high reliability from the huge number of unrelated pairs. However, even the best database searching procedures tested fail to find the large majority of distant evolutionary relationships at an acceptable error rate. Thus, if the procedures assessed here fail to find a reliable match, it does not imply that the sequence is unique; rather, it indicates that any relatives it might have are distant ones.\*\*

\*\*Additional and updated information about this work, including supplementary figures, may be found at <http://sss.stanford.edu/sss/>.

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## Differential gene expression in drug metabolism and toxicology: practicalities, problems and potential

JOHN C. ROCKETT†, DAVID J. ESDAILE‡  
and G. GORDON GIBSON\*

Molecular Toxicology Laboratory, School of Biological Sciences, University of Surrey,  
Guildford, Surrey, GU2 5XH, UK

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1. An important feature of the work of many molecular biologists is identifying which genes are switched on and off in a cell under different environmental conditions or subsequent to xenobiotic challenge. Such information has many uses, including the deciphering of molecular pathways and facilitating the development of new experimental and diagnostic procedures. However, the student of gene hunting should be forgiven for perhaps becoming confused by the mountain of information available as there appears to be almost as many methods of discovering differentially expressed genes as there are research groups using the technique.

2. The aim of this review was to clarify the main methods of differential gene expression analysis and the mechanistic principles underlying them. Also included is a discussion on some of the practical aspects of using this technique. Emphasis is placed on the so-called 'open' systems, which require no prior knowledge of the genes contained within the study model. Whilst these will eventually be replaced by 'closed' systems in the study of human, mouse and other commonly studied laboratory animals, they will remain a powerful tool for those examining less fashionable models.

3. The use of suppression-PCR subtractive hybridization is exemplified in the identification of up- and down-regulated genes in rat liver following exposure to phenobarbital, a well-known inducer of the drug metabolizing enzymes.

4. Differential gene display provides a coherent platform for building libraries and microchip arrays of 'gene fingerprints' characteristic of known enzyme inducers and xenobiotic toxicants, which may be interrogated subsequently for the identification and characterization of xenobiotics of unknown biological properties.

### Introduction

It is now apparent that the development of almost all cancers and many non-neoplastic diseases are accompanied by altered gene expression in the affected cells compared to their normal state (Hunter 1991, Wynford-Thomas 1991, Vogelstein and Kinzler 1993, Semenza 1994, Cassidy 1995, Kleinjan and Van Hegningen 1998). Such changes also occur in response to external stimuli such as pathogenic micro-organisms (Rohn *et al.* 1996, Singh *et al.* 1997, Griffin and Krishna 1998, Lunney 1998) and xenobiotics (Sewall *et al.* 1995, Dogra *et al.* 1998, Ramana and Kohli 1998), as well as during the development of undifferentiated cells (Hecht 1998, Rudin and Thompson 1998, Schneider-Maunoury *et al.* 1998). The potential medical and therapeutic benefits of understanding the molecular changes which occur in any given cell in progressing from the normal to the 'altered' state are enormous. Such profiling essentially provides a 'fingerprint' of each step of a

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\* Author for correspondence; e-mail: g.gibson@surrey.ac.uk

† Current Address: US Environmental Protection Agency, National Health and Environmental Effects, Research Laboratory, Reproductive Toxicology Division, Research Triangle Park, NC 27711, USA.

‡ Rhone-Poulenc Agrochemicals, Toxicology Department, Sophia-Antipolis, Nice, France.

cell's development or response and should help in the elucidation of specific and sensitive biomarkers representing, for example, different types of cancer or previous exposure to certain classes of chemicals that are enzyme inducers.

In drug metabolism, many of the xenobiotic-metabolizing enzymes (including the well-characterized isoforms of cytochrome P450) are inducible by drugs and chemicals in man (Pelkonen *et al.* 1998), predominantly involving transcriptional activation of not only the cognate cytochrome P450 genes, but additional cellular proteins which may be crucial to the phenomenon of induction. Accordingly, the development of methodology to identify and assess the full complement of genes that are either up- or down-regulated by inducers are crucial in the development of knowledge to understand the precise molecular mechanisms of enzyme induction and how this relates to drug action. Similarly, in the field of chemical-induced toxicity, it is now becoming increasingly obvious that most adverse reactions to drugs and chemicals are the result of multiple gene regulation, some of which are causal and some of which are casually-related to the toxicological phenomenon *per se*. This observation has led to an upsurge in interest in gene-profiling technologies which differentiate between the control and toxin-treated gene pools in target tissues and is, therefore, of value in rationalizing the molecular mechanisms of xenobiotic-induced toxicity. Knowledge of toxin-dependent gene regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. For example, if the gene profile in response to say a testicular toxin that has been well-characterized *in vivo* could be determined in the testis, then this profile would be representative of all new drug candidates which act via this specific molecular mechanism of toxicity, thereby providing a useful and coherent approach to the early detection of such toxicants. Whereas it would be informative to know the identity and functionality of all genes up/down regulated by such toxicants, this would appear a longer term goal, as the majority of human genes have not yet been sequenced, far less their functionality determined. However, the current use of gene profiling yields a *pattern* of gene changes for a xenobiotic of unknown toxicity which may be matched to that of well-characterized toxins, thus alerting the toxicologist to possible *in vivo* similarities between the unknown and the standard, thereby providing a platform for more extensive toxicological examination. Such approaches are beginning to gain momentum, in that several biotechnology companies are commercially producing 'gene chips' or 'gene arrays' that may be interrogated for toxicity assessment of xenobiotics. These chips consist of hundreds/thousands of genes, some of which are degenerate in the sense that not all of the genes are mechanistically-related to any one toxicological phenomenon. Whereas these chips are useful in broad-spectrum screening, they are maturing at a substantial rate, in that gene arrays are now becoming more specific, e.g. chips for the identification of changes in growth factor families that contribute to the aetiology and development of chemically-induced neoplasias.

Although documenting and explaining these genetic changes presents a formidable obstacle to understanding the different mechanisms of development and disease progression, the technology is now available to begin attempting this difficult challenge. Indeed, several 'differential expression analysis' methods have been developed which facilitate the identification of gene products that demonstrate



altered expression in cells of one population compared to another. These methods have been used to identify differential gene expression in many situations, including invading pathogenic microbes (Zhao *et al.* 1998), in cells responding to extracellular and intracellular microbial invasion (Duguid and Dinauer 1990, Ragno *et al.* 1997, Maldarelli *et al.* 1998), in chemically treated cells (Syed *et al.* 1997, Rockett *et al.* 1999), neoplastic cells (Liang *et al.* 1992, Chang and Terzaghi-Howe 1998), activated cells (Gurskaya *et al.* 1996, Wan *et al.* 1996), differentiated cells (Hara *et al.* 1991, Guimaraes *et al.* 1995a, b), and different cell types (Davis *et al.* 1984, Hedrick *et al.* 1984, Xhu *et al.* 1998). Although differential expression analysis technologies are applicable to a broad range of models, perhaps their most important advantage is that, in most cases, absolutely no prior knowledge of the specific genes which are up- or down-regulated is required.

The field of differential expression analysis is a large and complex one, with many techniques available to the potential user. These can be categorized into several methodological approaches, including:

- (1) Differential screening,
- (2) Subtractive hybridization (SH) (includes methods such as chemical cross-linking subtraction—CCLS, suppression-PCR subtractive hybridization—SSH, and representational difference analysis—RDA),
- (3) Differential display (DD),
- (4) Restriction endonuclease facilitated analysis (including serial analysis of gene expression—SAGE—and gene expression fingerprinting—GEF),
- (5) Gene expression arrays, and
- (6) Expressed sequence tag (EST) analysis.

The above approaches have been used successfully to isolate differentially expressed genes in different model systems. However, each method has its own subtle (and sometimes not so subtle) characteristics which incur various advantages and disadvantages. Accordingly, it is the purpose of this review to clarify the mechanistic principles underlying the main differential expression methods and to highlight some of the broader considerations and implications of this very powerful and increasingly popular technique. Specifically, we will concentrate on the so-called 'open' systems, namely those which do not require any knowledge of gene sequences and, therefore, are useful for isolating unknown genes. Two 'closed' systems (those utilising previously identified gene sequences), EST analysis and the use of DNA arrays, will also be considered briefly for completeness. Whilst emphasis will often be placed on suppression PCR subtractive hybridization (SSH, the approach employed in this laboratory), it is the aim of the authors to highlight, wherever possible, those areas of common interest to those who use, or intend to use, differential gene expression analysis.

### **Differential cDNA library screening (DS)**

Despite the development of multiple technological advances which have recently brought the field of gene expression profiling to the forefront of molecular analysis, recognition of the importance of differential gene expression and characterization of differentially expressed genes has existed for many years. One of the original approaches used to identify such genes was described 20 years ago by St John and Davis (1979). These authors developed a method, termed 'differential plaque filter

hybridization', which was used to isolate galactose-inducible DNA sequences from yeast. The theory is simple: a genomic DNA library is prepared from normal, unstimulated cells of the test organism/tissue and multiple filter replicas are prepared. These replica blots are probed with radioactively (or otherwise) labelled complex cDNA probes prepared from the control and test cell mRNA populations. Those mRNAs which are differentially expressed in the treated cell population will show a positive signal only on the filter probed with cDNA from the treated cells. Furthermore, labelled cDNA from different test conditions can be used to probe multiple blots, thereby enabling the identification of mRNAs which are only up-regulated under certain conditions. For example, St John and Davis (1979) screened replica filters with acetate-, glucose- and galactose-derived probes in order to obtain genes induced specifically by galactose metabolism. Although groundbreaking in its time this method is now considered insensitive and time-consuming, as up to 2 months are required to complete the identification of genes which are differentially expressed in the test population. In addition, there is no convenient way to check that the procedure has worked until the whole process has been completed.

### **Subtractive Hybridization (SH)**

The developing concept of differential gene expression and the success of early approaches such as that described by St John and Davis (1979) soon gave rise to a search for more convenient methods of analysis. One of the first to be developed was SH, numerous variations of which have since been reported (see below). In general, this approach involves hybridization of mRNA/cDNA from one population (tester) to excess mRNA/cDNA from another (driver), followed by separation of the unhybridized tester fraction (differentially expressed) from the hybridized common sequences. This step has been achieved physically, chemically and through the use of selective polymerase chain reaction (PCR) techniques.

#### *Physical separation*

Original subtractive hybridization technology involved the physical separation of hybridized common species from unique single stranded species. Several methods of achieving this have been described, including hydroxyapatite chromatography (Sargent and Dawid 1983), avidin-biotin technology (Duguid and Dinauer 1990) and oligodT-latex separation (Hara *et al.* 1991). In the first approach, common mRNA species are removed by cDNA (from test cells)-mRNA (from control cells) subtractive hybridization followed by hydroxyapatite chromatography, as hydroxyapatite specifically adsorbs the cDNA-mRNA hybrids. The unabsorbed cDNA is then used either for the construction of a cDNA library of differentially expressed genes (Sargent and Dawid 1983, Schneider *et al.* 1988) or directly as a probe to screen a preselected library (Zimmerman *et al.* 1980, Davis *et al.* 1984, Hedrick *et al.* 1984). A schematic diagram of the procedure is shown in figure 1.

Less rigorous physical separation procedures coupled with sensitivity enhancing PCR steps were later developed as a means to overcome some of the problems encountered with the hydroxyapatite procedure. For example, Duguid and Dinauer (1990) described a method of subtraction utilizing biotin-affinity systems as a means to remove hybridized common sequences. In this process, both the control and tester mRNA populations are first converted to cDNA and an adaptor ('oligovector',

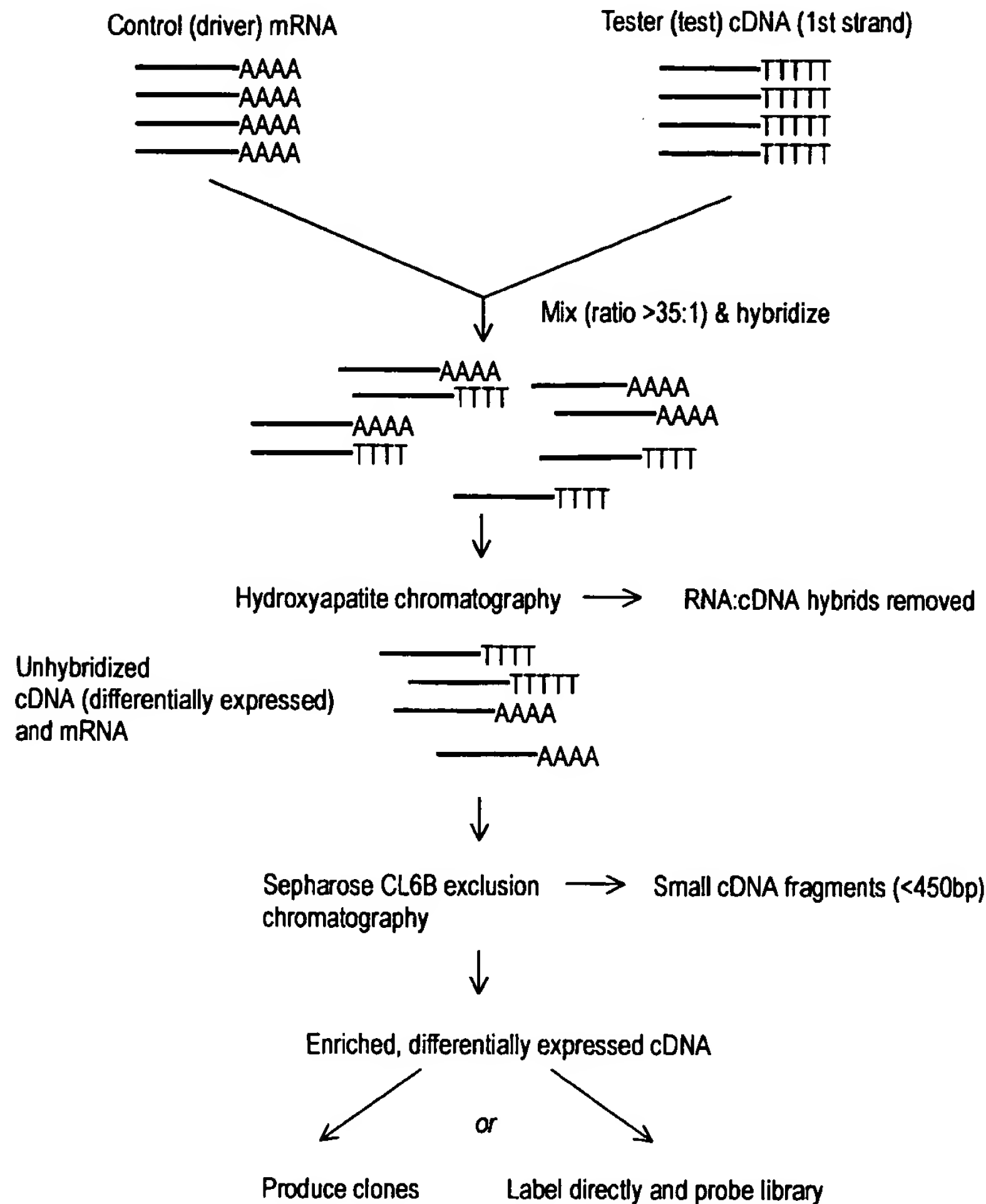


Figure 1. The hydroxyapatite method of subtractive hybridization. cDNA derived from the treated/alterd (tester) population is mixed with a large excess of mRNA from the control (driver) population. Following hybridization, mRNA-cDNA hybrids are removed by hydroxyapatite chromatography. The only cDNAs which remain are those which are differentially expressed in the treated/alterd population. In order to facilitate the recovery of full length clones, small cDNA fragments are removed by exclusion chromatography. The remaining cDNAs are then cloned into a vector for sequencing, or labelled and used directly to probe a library, as described by Sargent and Dawid (1983).

containing a restriction site) ligated to both sides. Both populations are then amplified by PCR, but the driver cDNA population is subsequently digested with the adaptor-containing restriction endonuclease. This serves to cleave the oligo-vector and reduce the amplification potential of the control population. The digested control population is then biotinylated and an excess mixed with tester cDNA. Following denaturation and hybridization, the mix is applied to a biocytin column (streptavidin may also be used) to remove the control population, including heteroduplexes formed by annealing of common sequences from the tester population. The procedure is repeated several times following the addition of fresh



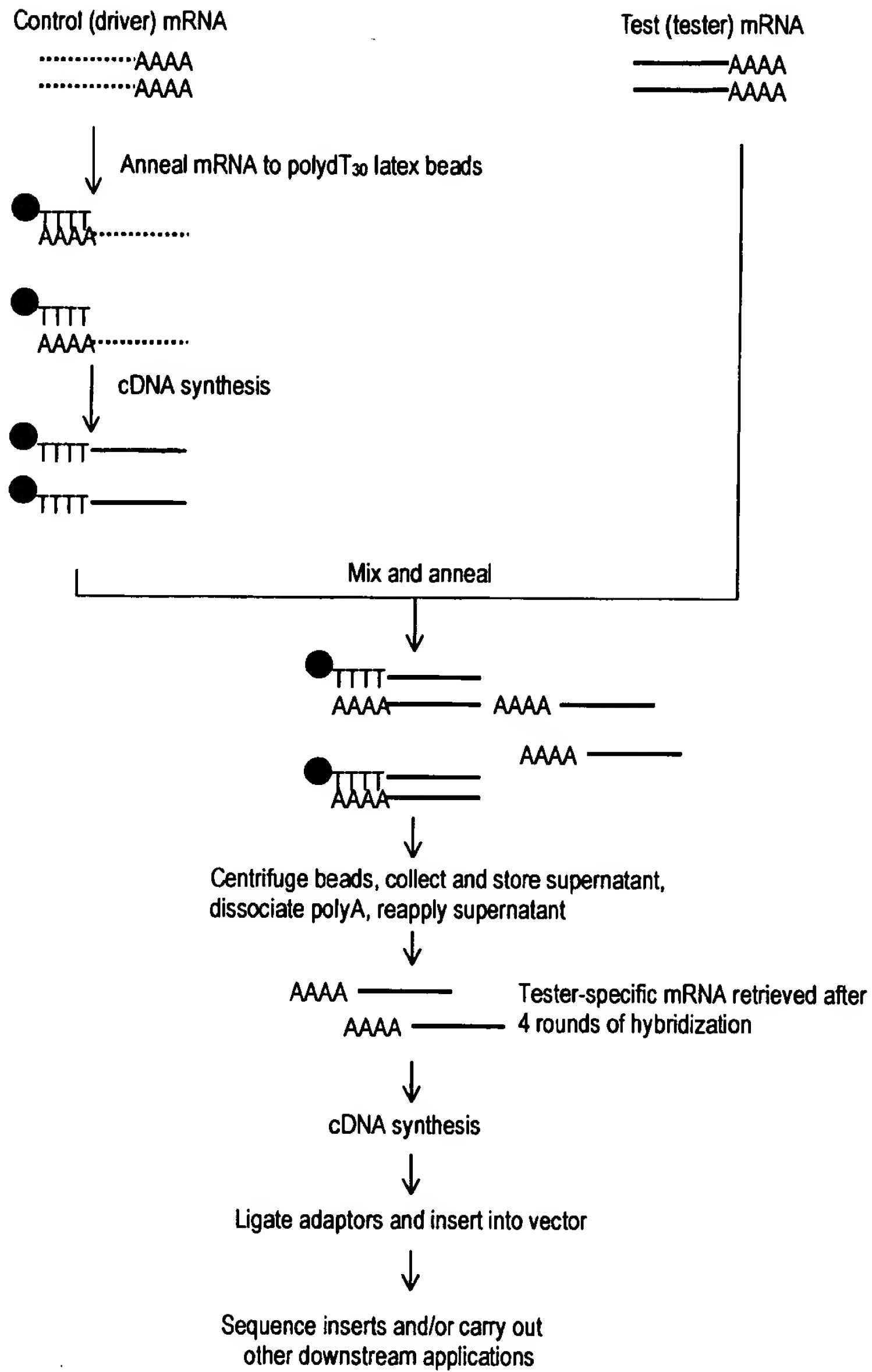


Figure 2. The use of oligodT<sub>30</sub> latex to perform subtractive hybridization. mRNA extracted from the control (driver) population is converted to anchored cDNA using polydT oligonucleotides attached to latex beads. mRNA from the treated/alterd (tester) population is repeatedly hybridized against an excess of the anchored driver cDNA. The final population of mRNA is tester specific and can be converted into cDNA for cloning and other downstream applications, as described by Hara *et al.* (1991).

control cDNA. In order to further enrich those species differentially expressed in the tester cDNA, the subtracted tester population is amplified by PCR following every second subtraction cycle. After six cycles of subtraction (three reamplification steps) the reaction mix is ligated into a vector for further analysis.

In a slightly different approach, Hara *et al.* (1991) utilized a method whereby oligo(dT<sub>30</sub>) primers attached to a latex substrate are used to first capture mRNA extracted from the control population. Following 1st strand cDNA synthesis, the RNA strand of the heteroduplexes is removed by heat denaturation and centrifugation (the cDNA-oligotex-dT<sub>30</sub> forms a pellet and the supernatant is removed). A quantity of tester mRNA is then repeatedly hybridized to the immobilized control (driver) cDNA (which is present in 20-fold excess). After several rounds of hybridization the only mRNA molecules left in the tester mRNA population are those which are not found in the driver cDNA-oligotex-dT<sub>30</sub> population. These tester-specific mRNA species are then converted to cDNA and, following the addition of adaptor sequences, amplified by PCR. The PCR products are then ligated into a vector for further analysis using restriction sites incorporated into the PCR primers. A schematic illustration of this subtraction process is shown in figure 2.

However, all these methods utilising physical separation have been described as inefficient due to the requirement for large starting amounts of mRNA, significant loss of material during the separation process and a need for several rounds of hybridization. Hence, new methods of differential expression analysis have recently been designed to eliminate these problems.

#### *Chemical Cross-Linking Subtraction (CCLS)*

In this technique, originally described by Hampson *et al.* (1992), driver mRNA is mixed with tester cDNA (1st strand only) in a ratio of > 20:1. The common sequences form cDNA:mRNA hybrids, leaving the tester specific species as single stranded cDNA. Instead of physically separating these hybrids, they are inactivated chemically using 2,5 diaziridiny-1,4-benzoquinone (DZQ). Labelled probes are then synthesized from the remaining single stranded cDNA species (unreacted mRNA species remaining from the driver are not converted into probe material due to specificity of Sequenase T7 DNA polymerase used to make the probe) and used to screen a cDNA library made from the tester cell population. A schematic diagram of the system is shown in figure 3.

It has been shown that the differentially expressed sequences can be enriched at least 300-fold with one round of subtraction (Hampson *et al.* 1992), and that the technique should allow isolation of cDNAs derived from transcripts that are present at less than 50 copies per cell. This equates to genes at the low end of intermediate abundance (see table 1). The main advantages of the CCLS approach are that it is rapid, technically simple and also produces fewer false positives than other differential expression analysis methods. However, like the physical separation protocols, a major drawback with CCLS is the large amount of starting material required (at least 10 µg RNA). Consequently, the technique has recently been refined so that a renewable source of RNA can be generated. The degenerate random oligonucleotide primed (DROP) adaptation (Hampson *et al.* 1996, Hampson and Hampson 1997) uses random hexanucleotide sequences to prime solid phase-synthesized cDNA. Since each primer includes a T7 polymerase promotor sequence

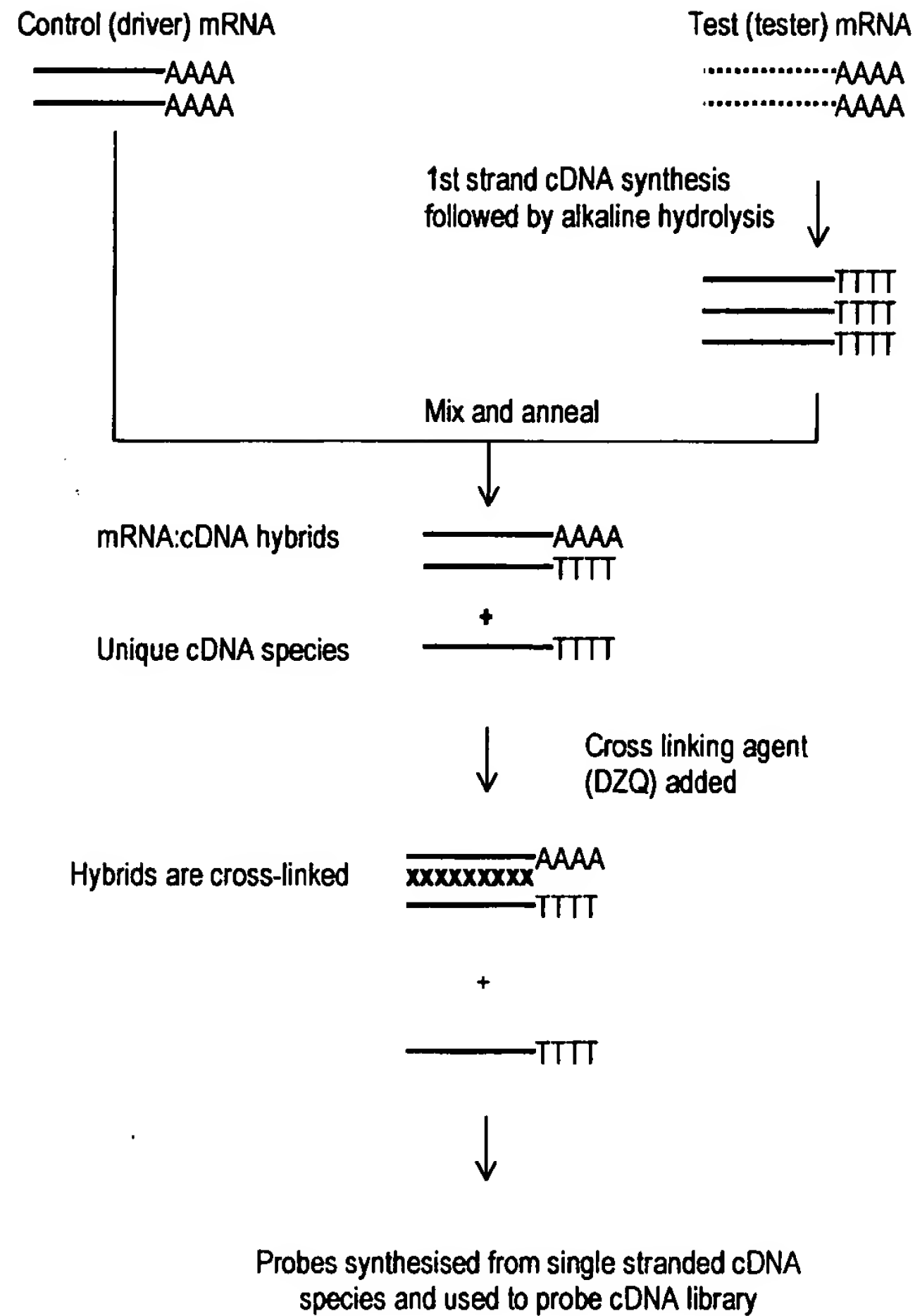


Figure 3. Chemical cross-linking subtraction. Excess driver mRNA is mixed with 1<sup>st</sup> strand tester cDNA. The common sequences form mRNA:cDNA hybrids which are cross linked with 2,5 diaziridinyl-1,4-benzoquinone (DZQ) and the remaining cDNA sequences are differentially expressed in the tester population. Probes are made from these sequences using Sequenase 2.0 DNA polymerase, which lacks reverse transcriptase activity and, therefore, does not react with the remaining mRNA molecules from the driver. The labelled probes are then used to screen a cDNA library for clones of differentially expressed sequences. Adapted from Walter *et al.* (1996), with permission.

Table 1. The abundance of mRNA species and classes in a typical mammalian cell.

mRNA class	Copies of each species/cell	No. of mRNA species in class	Mean % of each species in class	Mean mass (ng) of each species/ $\mu$ g total RNA
Abundant	12000	4	3.3	1.65
Intermediate	300	500	0.08	0.04
Rare	15	11000	0.004	0.002

Modified from Bertoli *et al.* (1995).



at the 5' end, the final pool of random cDNA fragments is a PCR-renewable cDNA population which is representative of the expressed gene pool and can be used to synthesize sense RNA for use as driver material. Furthermore, if the final pool of random cDNA fragments is reamplified using biotinylated T7 primer and random hexamer, the product can be captured with streptavidin beads and the antisense strand eluted for use as tester. Since both target and driver can be generated from the same DROP product, subtraction can be performed in both directions (i.e. for up- and down-regulated species) between two different DROP products.

#### *Representational Difference Analysis (RDA)*

RDA of cDNA (Hubank and Schatz 1994) is an extension of the technique originally applied to genomic DNA as a means of identifying differences between two complex genomes (Lisitsyn *et al.* 1993). It is a process of subtraction and amplification involving subtractive hybridization of the tester in the presence of excess driver. Sequences in the tester that have homologues in the driver are rendered unamplifiable, whereas those genes expressed only in the tester retain the ability to be amplified by PCR. The procedure is shown schematically in figure 4.

In essence, the driver and tester mRNA populations are first converted to cDNA and amplified by PCR following the ligation of an adaptor. The adaptors are then removed from both populations and a new (different) adaptor ligated to the amplified tester population only. Driver and tester populations are next melted and hybridized together in a ratio of 100:1. Following hybridization, only tester:tester homohybrids have 5' adaptors at each end of the DNA duplex and can, thus, be filled in at both 3' ends. Hence, only these molecules are amplified exponentially during the subsequent PCR step. Although tester:driver heterohybrids are present, they only amplify in a linear fashion, since the strand derived from the driver has no adaptor to which the primer can bind. Driver:driver heterohybrids have no adaptors and, therefore, are not amplified. Single stranded molecules are digested with mung bean nuclease before a further PCR-enrichment of the tester:tester homohybrids. The adaptors on the amplified tester population are then replaced and the whole process repeated a further two or three times using an increasing excess of driver (Hubank and Schatz used a tester:driver ratio of 1:400, 1:80000 and 1:800000 for the second, third and fourth hybridizations, respectively). Different adaptors are ligated to the tester between successive rounds of hybridization and amplification to prevent the accumulation of PCR products that might interfere with subsequent amplifications. The final display is a series of differentially expressed gene products easily observable on an ethidium bromide gel.

The main advantages of RDA are that it offers a reproducible and sensitive approach to the analysis of differentially expressed genes. Hubank and Schatz (1994) reported that they were able to isolate genes that were differentially expressed in substantially less than 1% of the cells from which the tester is derived. Perhaps the main drawback is that multiple rounds of ligation, hybridization, amplification and digestion are required. The procedure is, therefore, lengthier than many other differential display approaches and provides more opportunity for operator-induced error to occur. Although the generation of false positives has been noted, this has been solved to some degree by O'Neill and Sinclair (1997) through the use of HPLC-purified adaptors. These are free of the truncated adaptors which appear to be a major source of the false positive bands. A very similar technique to RDA, termed linker capture subtraction (LCS) was described by Yang and Sytowski (1996).

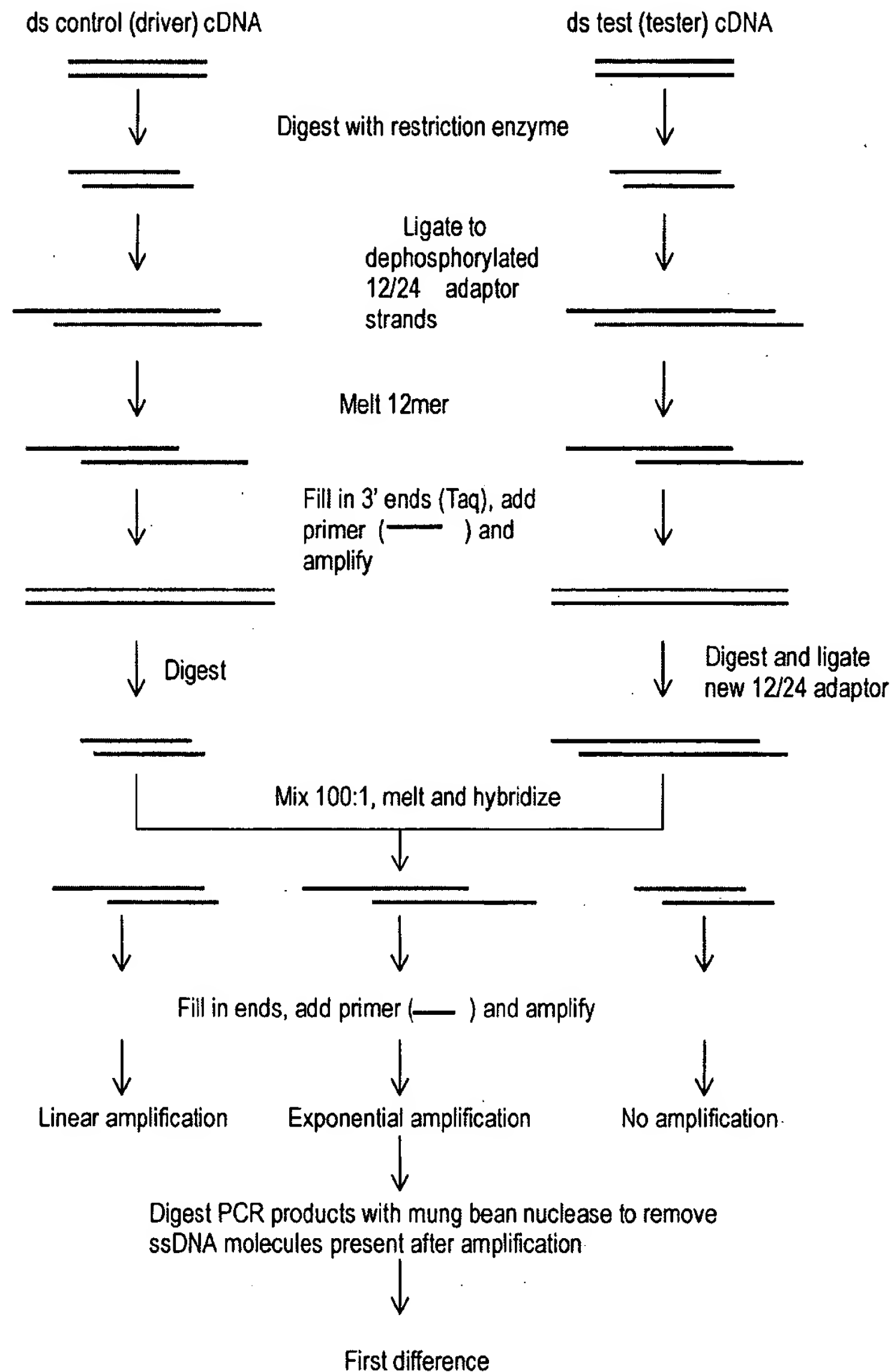


Figure 4. The representational difference analysis (RDA) technique. Driver and tester cDNA are digested with a 4-cutter restriction enzyme such as *DpnII*. The 1<sup>st</sup> set of 12/24 adaptor strands (oligonucleotides) are ligated to each other and the digested cDNA products. The 12mer is subsequently melted away and the 3' ends filled in using Taq DNA polymerase. Each cDNA population is then amplified using PCR, following which the 1<sup>st</sup> set of adaptors is removed with *DpnII*. A second set of 12/24 adaptor strands is then added to the amplified tester cDNA population, after which the tester is hybridized against a large excess of driver. The 12mer adaptors are melted and the 3' ends filled in as before. PCR is carried out with primers identical to the new 24mer adaptor. Thus, the only hybridization products which are exponentially amplified are those which are tester:tester combinations. Following PCR, ssDNA products are removed with mung bean nuclease, leaving the 'first difference product'. This is digested and a third set of 12/24 adaptors added before repeating the subtraction process from the hybridization stage. The process is repeated to the 3<sup>rd</sup> or 4<sup>th</sup> difference product, as described by Lisitsyn *et al.* (1993) and Hubank and Schatz (1994).

*Suppression PCR Subtractive Hybridization (SSH)*

The most recent adaptation of the SH approach to differential expression analysis was first described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996). They reported that a 1000–5000 fold enrichment of rare cDNAs (equivalent to isolating mRNAs present at only a few copies per cell) can be obtained without the need for multiple hybridizations/subtractions. Instead of physical or chemical removal of the common sequences, a PCR-based suppression system is used (see figure 5).

In SSH, excess driver cDNA is added to two portions of the tester cDNA which have been ligated with different adaptors. A first round of hybridization serves to enrich differentially expressed genes and equalize rare and abundant messages. Equalization occurs since reannealing is more rapid for abundant molecules than for rarer molecules due to the second order kinetics of hybridization (James and Higgins 1985). The two primary hybridization mixes are then mixed together in the presence of excess driver and allowed to hybridize further. This step permits the annealing of single stranded complementary sequences which did not hybridize in the primary hybridization, and in doing so generates templates for PCR amplification. Although there are several possible combinations of the single stranded molecules present in the secondary hybridization mix, only one particular combination (differentially expressed in the tester cDNA composed of complimentary strands having different adaptors) can amplify exponentially.

Having obtained the final differential display, two options are available if cloning of cDNAs is desired. One is to transform the whole of the final PCR reaction into competent cells. Transformed colonies can then be isolated and their inserts characterized by sequencing, restriction analysis or PCR. Alternatively, the final PCR products can be resolved on a gel and the individual bands excised, reamplified and cloned. The first approach is technically simpler and less time consuming. However, ligation/transformation reactions are known to be biased towards the cloning of smaller molecules, and so the final population of clones will probably not contain a representative selection of the larger products. In addition, although equalization theoretically occurs, observations in this laboratory suggest that this is by no means perfectly accomplished. Consequently, some gene species are present in a higher number than others and this will be represented in the final population of clones. Thus, in order to obtain a substantial proportion of those gene species that actually demonstrate differential expression in the tester population, the number of clones that will have to be screened after this step may be substantial. The second approach is initially more time consuming and technically demanding. However, it would appear to offer better prospects for cloning larger and low abundance gel products. In addition, one can incorporate a screening step that differentiates different products of different sequences but of the same size (HA-staining, see later). In this way, a good idea of the final number of clones to be isolated and identified can be achieved.

An alternative (or even complementary) approach is to use the final differential display reaction to screen a cDNA library to isolate full length clones for further characterization, or a DNA array (see later) to quickly identify known genes. SSH has been used in this laboratory to begin characterization of the short-term gene expression profiles of enzyme-inducers such as phenobarbital (Rockett *et al.* 1997) and Wy-14,643 (Rockett *et al.* unpublished observations). The isolation of differentially expressed genes in this manner enables the construction of a fingerprint



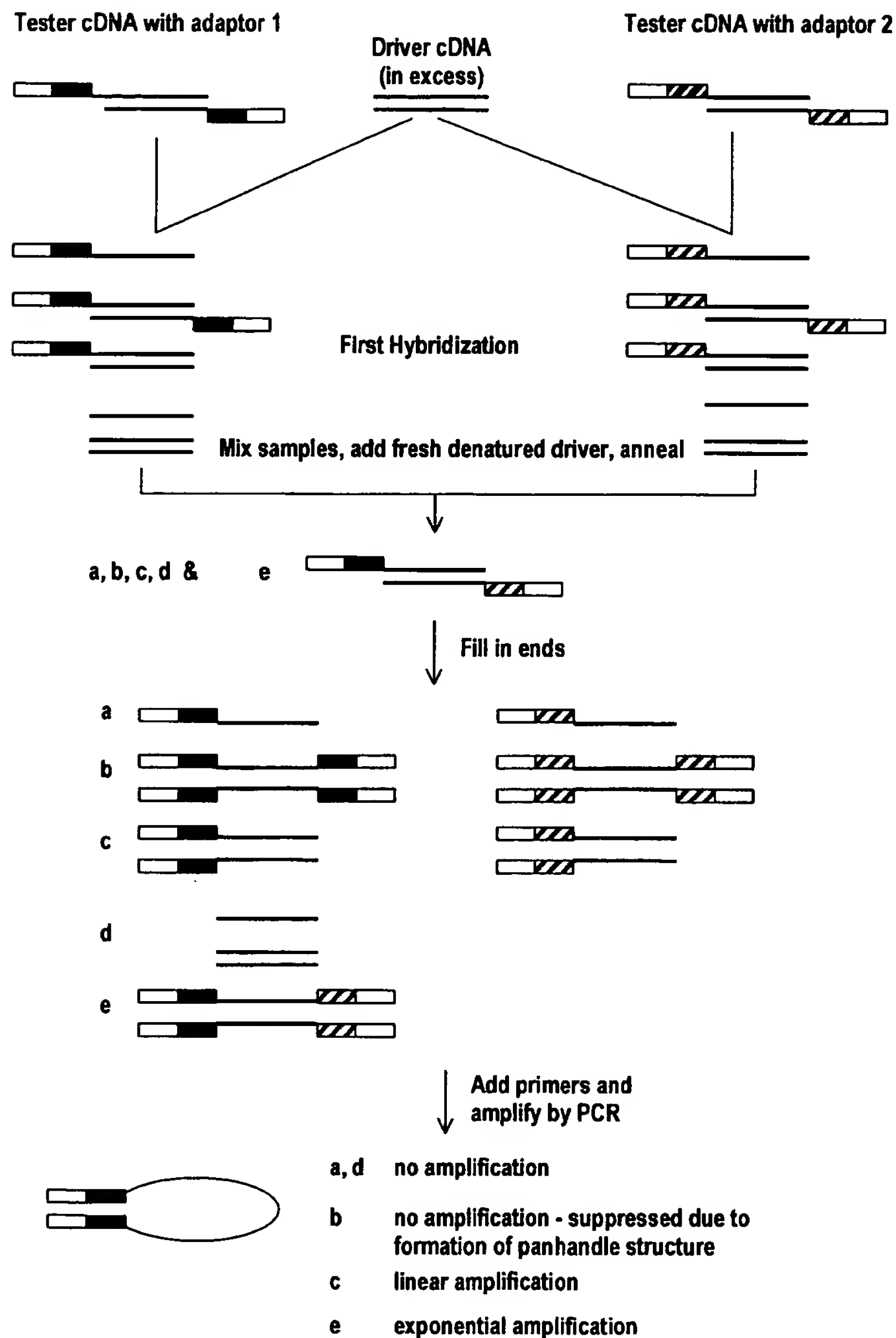


Figure 5. PCR-select cDNA subtraction. In the primary hybridization, an excess of driver cDNA is added to each tester cDNA population. The samples are heat denatured and allowed to hybridize for between 3 and 8 h. This serves two purposes: (1) to equalize rare and abundant molecules; and (2) to enrich for differentially expressed sequences—cDNAs that are not differentially expressed form type c molecules with the driver. In the secondary hybridization, the two primary hybridizations are mixed together without denaturing. Fresh denatured driver can also be added at this point to allow further enrichment of differentially expressed sequences. Type e molecules are formed in this secondary hybridization which are subsequently amplified using two rounds of PCR. The final products can be visualized on an agarose gel, labelled directly or cloned into a vector for downstream manipulation. As described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996), with permission.

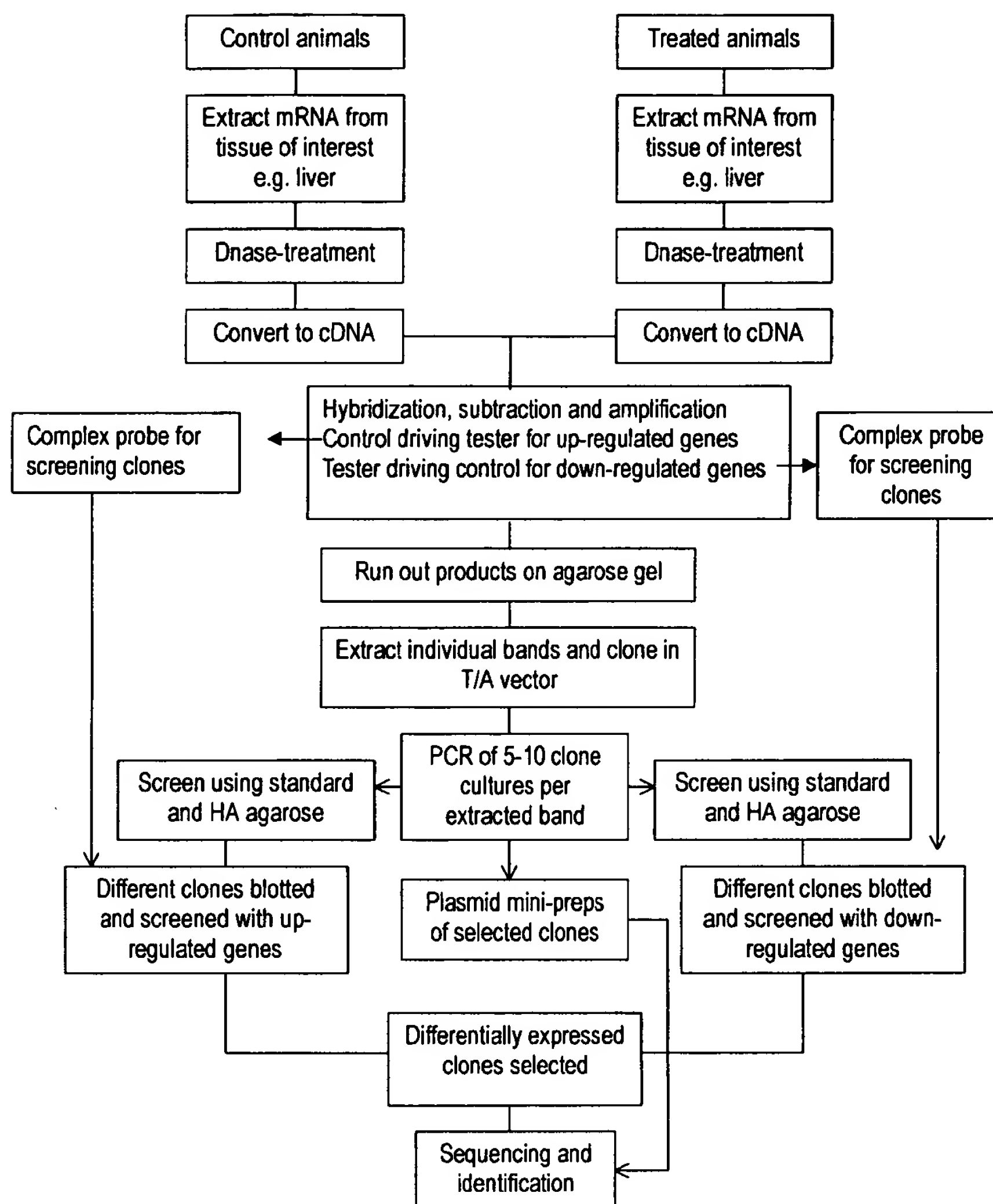


Figure 6. Flow diagram showing method used in this laboratory to isolate and identify clones of genes which are differentially expressed in rat liver following short term exposure to the enzyme inducers, phenobarbital and Wy-14,643.

of expressed genes which are unique to each compound and time/dose point. Such information could be useful in short-term characterization of the toxic potential of new compounds by comparing the gene-expression profiles they elicit with those produced by known inducers. Figure 6 shows a flow diagram of the method used to isolate, verify and clone differentially expressed genes, and figure 7 shows expression profiles obtained from a typical SSH experiment. Subsequent sub-cloning of the individual bands, sequencing and gene data base interrogation reveals many genes which are either up- or down-regulated by phenobarbital in the rat (tables 2 and 3).

One of the advantages in using the SSH approach is that no prior knowledge is required of which specific genes are up/down-regulated subsequent to xenobiotic

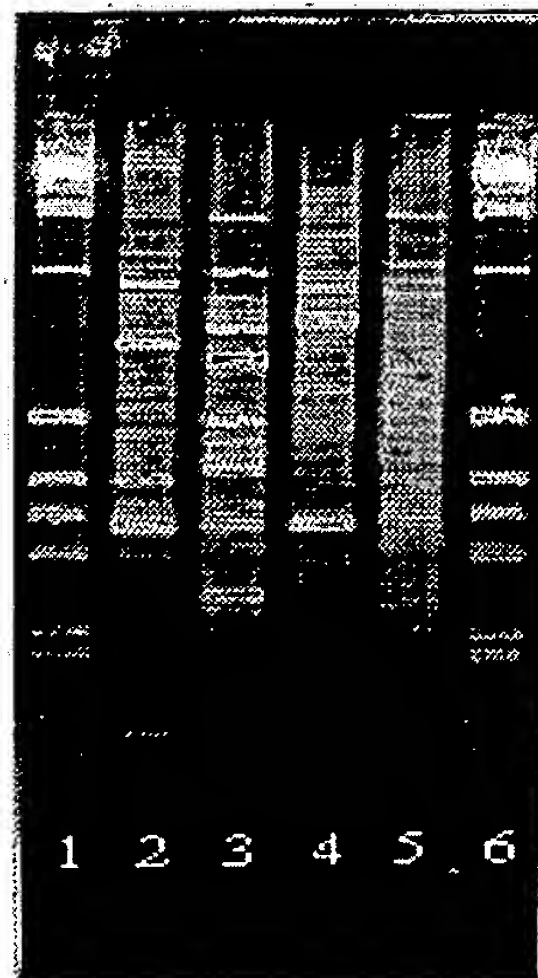


Figure 7. SSH display patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane: 1—1kb ladder; 2—genes upregulated following Wy,14-643 treatment; 3—genes downregulated following Wy,14-643 treatment; 4—genes upregulated following phenobarbital treatment; 5—genes downregulated following phenobarbital treatment; 6—1kb ladder. Reproduced from Rockett *et al.* (1997), with permission.

exposure, and an almost complete complement of genes are obtained. For example, the peroxisome proliferator and non-genotoxic hepatocarcinogen Wy,14,643, up-regulates at least 28 genes and down-regulates at least 15 in the rat (a sensitive species) and produces 48 up- and 37 down-regulated genes in the guinea pig, a resistant species (Rockett, Swales, Esda and Gibson, unpublished observations). One of these genes, CD81, was up-regulated in the rat and down-regulated in the guinea pig following Wy-14,643 treatment. CD81 (alternatively named TAPA-1) is a widely expressed cell surface protein which is involved in a large number of cellular processes including adhesion, activation, proliferation and differentiation (Levy *et al.* 1998). Since all of these functions are altered to some extent in the phenomena of hepatomegaly and non-genotoxic hepatocarcinogenesis, it is intriguing, and probably mechanistically-relevant, that CD81 expression is differentially regulated in a resistant and susceptible species. However, the down-side of this approach is that the majority of genes can be sequenced and matched to database sequences, but the latter are predominantly expressed sequence tags or genes of completely unknown function, thus partially obscuring a realistic overall assessment of the critical genes of genuine biological interest. Notwithstanding the lack of complete functional identification of altered gene expression, such gene profiling studies essentially provides a 'molecular fingerprint' in response to xenobiotic challenge, thereby serving as a mechanistically-relevant platform for further detailed investigations.

#### Differential Display (DD)

Originally described as 'RNA fingerprinting by arbitrarily primed PCR' (Liang and Pardee 1992) this method is now more commonly referred to as 'differential



Table 2. Genes up-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
5 (1300)	93.5%	CYP2B1
7 (1000)	95.1%	Preproalbumin Serum albumin mRNA
8 (950)	98.3%	NCI-CGAP-Pr1 <i>H. sapiens</i> (EST)
10 (850)	95.7%	CYP2B1
11 (800)	Clone 1 94.9%	CYP2B1
	Clone 2 75.3%	CYP2B2
12 (750)	93.8%	TRPM-2 mRNA Sulfated glycoprotein
15 (600)	92.9%	Preproalbumin Serum albumin mRNA
16 (55)	Clone 1 95.2%	CYP2B1
	Clone 2 93.6%	Haptoglobin mRNA partial alpha
21 (350)	99.3%	18S, 5.8S & 28S rRNA

Bands 1–4, 6, 9, 13, 14, and 17–20 are shown to be false positives by dot blot analysis and, therefore, are not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are up-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

Table 3. Genes down-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
1 (1500)	95.3%	3-oxoacyl-CoA thiolase
2 (1200)	92.3%	Hemopoxin mRNA
3 (1000)	91.7%	Alpha-2u-globulin mRNA
7 (700)	Clone 1 77.2%	<i>M. musculus</i> C1 inhibitor
	Clone 2 94.5%	Electron transfer flavoprotein
	Clone 3 91.0%	<i>M. musculus</i> Topoisomerase 1 (Topo 1)
8 (650)	Clone 1 86.9%	Soares 2NbMT <i>M. musculus</i> (EST)
	Clone 2 96.2%	Alpha-2u-globulin (s-type) mRNA
9 (600)	Clone 1 86.9%	Soares mouse NML <i>M. musculus</i> (EST)
	Clone 2 82.0%	Soares p3NMF 19.5 <i>M. musculus</i> (EST)
10 (550)	73.8%	Soares mouse NML <i>M. musculus</i> (EST)
11 (525)	95.7%	NCI-CGAP-Pr1 <i>H. sapiens</i> (EST)
12 (375)	100.0%	Ribosomal protein
13 (23)	Clone 1 97.2%	Soares mouse embryo NbME135 (EST)
	Clone 2 100.0%	Fibrinogen B-beta-chain
	Clone 3 100.0%	Apolipoprotein E gene
14 (170)	96.0%	Soares p3NMF19.5 <i>M. musculus</i> (EST)
15 (140)	97.3%	Stratagene mouse testis (EST)
Others: (300)	96.7%	<i>R. norvegicus</i> RASP 1 mRNA
(275)	93.1%	Soares mouse mammary gland (EST)

EST = Expressed sequence tag. Bands 4–6 were shown to be false positives by dot blot analysis and, therefore, were not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are down-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

display' (DD). In this method, all the mRNA species in the control and treated cell populations are amplified in separate reactions using reverse transcriptase-PCR (RT-PCR). The products are then run side-by-side on sequencing gels. Those bands which are present in one display only, or which are much more intense in one

display compared to the other, are differentially expressed and may be recovered for further characterization. One advantage of this system is the speed with which it can be carried out—2 days to obtain a display and as little as a week to make and identify clones.

Two commonly used variations are based on different methods of priming the reverse transcription step (figure 8). One is to use an oligo dT with a 2-base 'anchor' at the 3'-end, e.g. 5' (dT<sub>11</sub>)CA 3' (Liang and Pardee 1992). Alternatively, an arbitrary primer may be used for 1st strand cDNA synthesis (Welsh *et al.* 1992). This variant of RNA fingerprinting has also been called 'RAP' (RNA Arbitrarily Primed)-PCR. One advantage of this second approach is that PCR products may be derived from anywhere in the RNA, including open reading frames. In addition, it can be used for mRNAs that are not polyadenylated, such as many bacterial mRNAs (Wong and McClelland 1994). In both cases, following reverse transcription and denaturation, second strand cDNA synthesis is carried out with an arbitrary primer (*arbitrary* primers have a single base at each position, as compared to *random* primers, which contain a mixture of all four bases at each position). The resulting PCR, thus, produces a series of products which, depending on the system (primer length and composition, polymerase and gel system), usually includes 50–100 products per primer set (Band and Sager 1989). When a combination of different dT-anchors and arbitrary primers are used, almost all mRNA species from a cell can be amplified. When the cDNA products from two different populations are analysed side by side on a polyacrylamide gel, differences in expression can be identified and the appropriate bands recovered for cloning and further analysis.

Although DD is perhaps the most popular approach used today for identifying differentially expressed genes, it does suffer from several perceived disadvantages:

- (1) It may have a strong bias towards high copy number mRNAs (Bertioli *et al.* 1995), although this has been disputed (Wan *et al.* 1996) and the isolation of very low abundance genes may be achieved in certain circumstances (Guimeraes *et al.* 1995a).
- (2) The cDNAs obtained often only represent the extreme 3' end of the mRNA (often the 3'-untranslated region), although this may not always be the case (Guimeraes *et al.* 1995a). Since the 3' end is often not included in Genbank and shows variation between organisms, cDNAs identified by DD cannot always be matched with their genes, even if they have been identified.
- (3) The pattern of differential expression seen on the display often cannot be reproduced on Northern blots, with false positives arising in up to 70% of cases (Sun *et al.* 1994). Some adaptations have been shown to reduce false positives, including the use of two reverse transcriptases (Sung and Denman 1997), comparison of uninduced and induced cells over a time course (Burn *et al.* 1994) and comparison of DDPCR-products from two uninduced and two induced lines (Sompayrac *et al.* 1995). The latter authors also reported that the use of cytoplasmic RNA rather than total RNA reduces false positives arising from nuclear RNA that is not transported to the cytoplasm.

Further details of the background, strengths and weaknesses of the DD technique can be obtained from a review by McClelland *et al.* (1996) and from articles by Liang *et al.* (1995) and Wan *et al.* (1996).

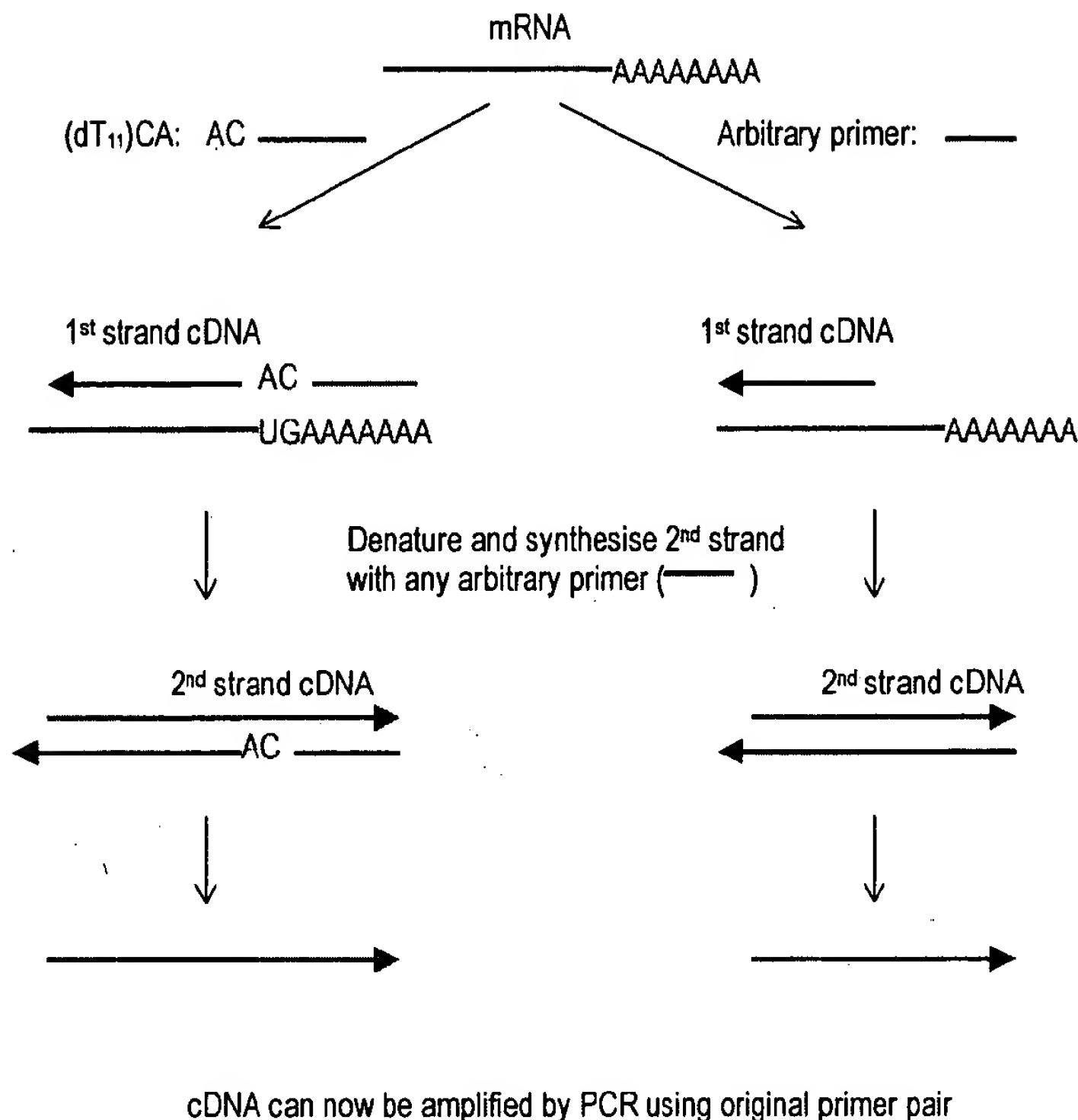


Figure 8. Two approaches to differential display (DD) analysis. 1<sup>st</sup> strand synthesis can be carried out either with a polydT<sub>11</sub>NN primer (where N = G, C or A) or with an arbitrary primer. The use of different combinations of G, C and A to anchor the first strand polydT primer enables the priming of the majority of polyadenylated mRNAs. Arbitrary primers may hybridize at none, one or more places along the length of the mRNA, allowing 1<sup>st</sup> strand cDNA synthesis to occur at none, one or more points in the same gene. In both cases, 2<sup>nd</sup> strand synthesis is carried out with an arbitrary primer. Since these arbitrary primers for the 2<sup>nd</sup> strand may also hybridize to the 1<sup>st</sup> strand cDNA in a number of different places, several different 2<sup>nd</sup> strand products may be obtained from one binding point of the 1<sup>st</sup> strand primer. Following 2<sup>nd</sup> strand synthesis, the original set of primers is used to amplify the second strand products, with the result that numerous gene sequences are amplified.

### Restriction endonuclease-facilitated analysis of gene expression

#### *Serial Analysis of Gene Expression (SAGE)*

A more recent development in the field of differential display is SAGE analysis (Velculescu *et al.* 1995). This method uses a different approach to those discussed so far and is based on two principles. Firstly, in more than 95% of cases, short nucleotide sequences ('tags') of only nine or 10 base pairs provide sufficient information to identify their gene of origin. Secondly, concatenation (linking together in a series) of these tags allows sequencing of multiple cDNAs within a single clone. Figure 9 shows a schematic representation of the SAGE process. In this procedure, double stranded cDNA from the test cells is synthesized with a biotinylated polydT primer. Following digestion with a commonly cutting (4bp recognition sequence) restriction enzyme ('anchoring enzyme'), the 3' ends of the cDNA population are captured with streptavidin beads. The captured population is



split into two and different adaptors ligated to the 5' ends of each group. Incorporated into the adaptors is a recognition sequence for a type IIS restriction enzyme—one which cuts DNA at a defined distance (< 20 bp) from its recognition sequence. Hence, following digestion of each captured cDNA population with the IIS enzyme, the adaptors plus a short piece of the captured cDNA are released. The two populations are then ligated and the products amplified. The amplified products are cleaved with the original anchoring enzyme, religated (concatomers are formed in the process) and cloned. The advantage of this system is that hundreds of gene tags can be identified by sequencing only a few clones. Furthermore, the number of times a given transcript is identified is a quantitative measurement of that gene's abundance in the original population, a feature which facilitates identification of differentially expressed genes in different cell populations.

Some disadvantages of SAGE analysis include the technical difficulty of the method, a large amount of accurate sequencing is required, biased towards abundant mRNAs, has not been validated in the pharmaco/toxicogenomic setting and has only been used to examine well known tissue differences to date.

#### *Gene Expression Fingerprinting (GEF)*

A different capture/restriction digest approach for isolating differentially expressed genes has been described by Ivanova and Belyavsky (1995). In this method, RNA is converted to cDNA using biotinylated oligo(dT) primers. The cDNA population is then digested with a specific endonuclease and captured with magnetic streptavidin microbeads to facilitate removal of the unwanted 5' digestion products. The use of restricted 3'-ends alone serves to reduce the complexity of the cDNA fragment pool and helps to ensure that each RNA species is represented by not more than one restriction product. An adaptor is ligated to facilitate subsequent amplification of the captured population. PCR is carried out with one adaptor-specific and one biotinylated polydT primer. The reamplified population is recaptured and the non-biotinylated strands removed by alkaline dissociation. The non-biotinylated strand is then resynthesized using a different adaptor-specific primer in the presence of a radiolabelled dNTP. The labelled immobilized 3' cDNA ends are next sequentially treated with a series of different restriction endonucleases and the products from each digestion analysed by PAGE. The result is a fingerprint composed of a number of ladders (equal to the number of sequential digests used). By comparing test versus control fingerprints, it is possible to identify differentially expressed products which can then be isolated from the gel and cloned. The advantages of this procedure are that it is very robust and reproducible, and the authors estimate that 80–93% of cDNA molecules are involved in the final fingerprint. The disadvantage is that polyacrylamide gels can rarely resolve more than 300–400 bands, which compares poorly to the 1000 or more which are estimated to be produced in an average experiment. The use of 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991) may help to overcome this problem.

A similar method for displaying restriction endonuclease fragments was later described by Prashar and Weissman (1996). However, instead of sequential digestion of the immobilized 3'-terminal cDNA fragments, these authors simply compared the profiles of the control and treated populations without further manipulation.

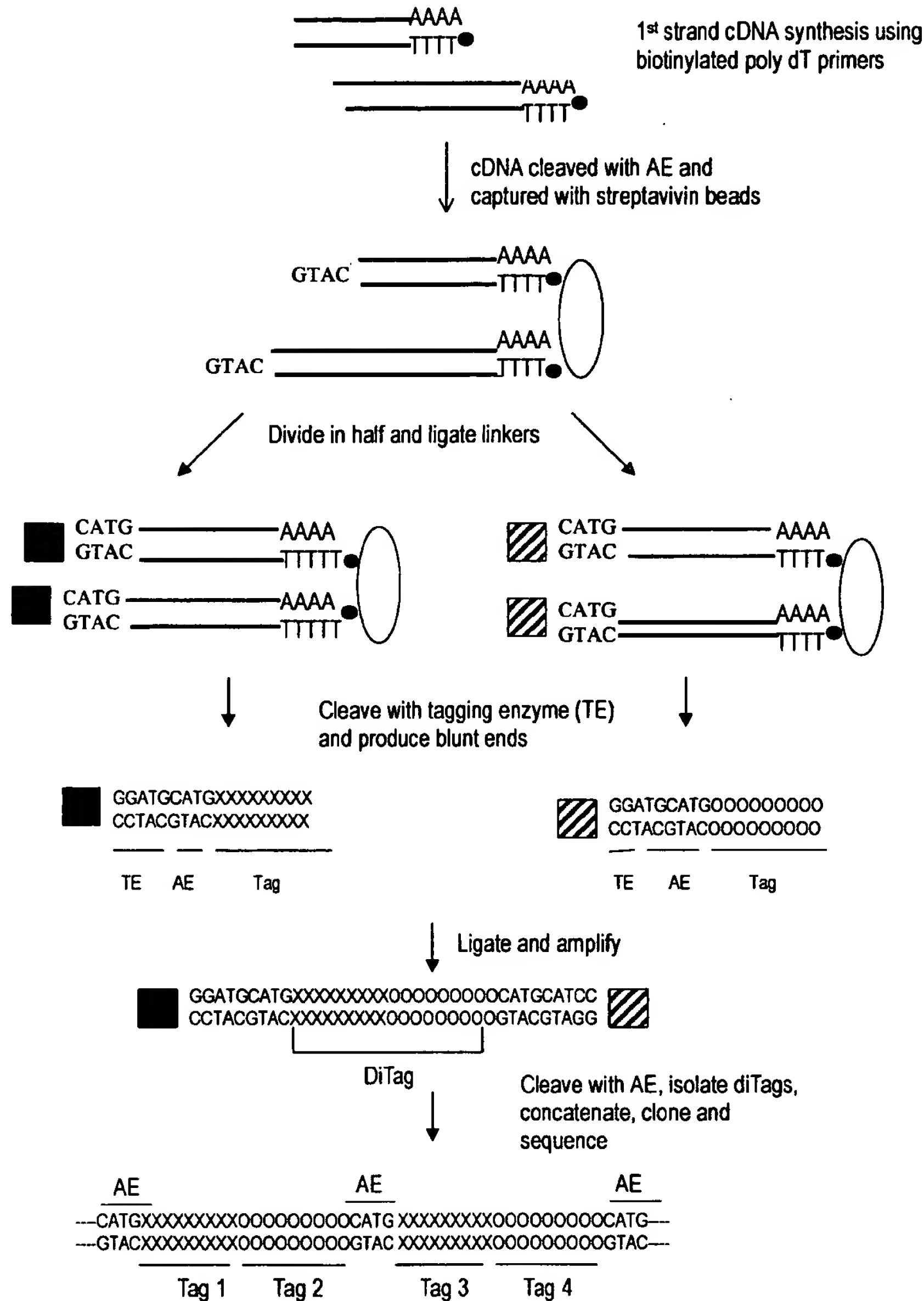


Figure 9. Serial analysis of gene expression (SAGE) analysis. cDNA is cleaved with an anchoring enzyme (AE) and the 3' ends captured using streptavidin beads. The cDNA pool is divided in half and each portion ligated to a different linker, each containing a type IIS restriction site (tagging enzyme, TE). Restriction with the type IIS enzyme releases the linker plus a short length of cDNA (XXXXX and OOOOO indicate nucleotides of different tags). The two pools of tags are then ligated and amplified using linker-specific primers. Following PCR, the products are cleaved with the AE and the ditags isolated from the linkers using PAGE. The ditags are then ligated (during which process, concatenization occurs) and cloned into a vector of choice for sequencing. After Velculescu *et al.* (1995), with permission.

### DNA arrays

'Open' differential display systems are cumbersome in that it takes a great deal of time to extract and identify candidate genes and then confirm that they are indeed up- or down-regulated in the treated compared to the control tissue. Normally, the latter process is carried out using Northern blotting or RT-PCR. Even so, each of the aforementioned steps produce a bottleneck to the ultimate goal of rapid analysis of gene expression. These problems will likely be addressed by the development of so-called DNA arrays (e.g. Gress *et al.* 1992, Zhao *et al.* 1995, Schena *et al.* 1996), the introduction of which has signalled the next era in differential gene expression analysis. DNA arrays consist of a gridded membrane or glass 'chips' containing hundreds or thousands of DNA spots, each consisting of multiple copies of part of a known gene. The genes are often selected based on previously proven involvement in oncogenesis, cell cycling, DNA repair, development and other cellular processes. They are usually chosen to be as specific as possible for each gene and animal species. Human and mouse arrays are already commercially available and a few companies will construct a personalized array to order, for example Clontech Laboratories and Research Genetics Inc. The technique is rapid in that hundreds or even thousands of genes can be spotted on a single array, and that mRNA/cDNA from the test populations can be labelled and used directly as probe. When analysed with appropriate hardware and software, arrays offer a rapid and quantitative means to assess differences in gene expression between two cell populations. Of course, there can only be identification and quantitation of those genes which are in the array (hence the term 'closed' system). Therefore, one approach to elucidating the molecular mechanisms involved in a particular disease/development system may be to combine an open and closed system—a DNA array to directly identify and quantitate the expression of known genes in mRNA populations, and an open system such as SSH to isolate unknown genes which are differentially expressed.

One of the main advantages of DNA arrays is the huge number of gene fragments which can be put on a membrane—some companies have reported gridding up to 60000 spots on a single glass 'chip' (microscope slide). These high density chip-based micro-arrays will probably become available as mass-produced off-the-shelf items in the near future. This should facilitate the more rapid determination of differential expression in time and dose-response experiments. Aside from their high cost and the technical complexities involved in producing and probing DNA arrays, the main problem which remains, especially with the newer micro-array (gene-chip) technologies, is that results are often not wholly reproducible between arrays. However, this problem is being addressed and should be resolved within the next few years.

### EST databases as a means to identify differentially expressed genes

Expressed sequence tags (ESTs) are partial sequences of clones obtained from cDNA libraries. Even though most ESTs have no formal identity (putative identification is the best to be hoped for), they have proven to be a rapid and efficient means of discovering new genes and can be used to generate profiles of gene-expression in specific cells. Since they were first described by Adams *et al.* (1991), there has been a huge explosion in EST production and it is estimated that there are now well over a million such sequences in the public domain, representing over half



of all human genes (Hillier *et al.* 1996). This large number of freely available sequences (both sequence information and clones are normally available royalty-free from the originators) has enabled the development of a new approach towards differential gene expression analysis as described by Vasmataz *et al.* (1998). The approach is simple in theory: EST databases are first searched for genes that have a number of related EST sequences from the target tissue of choice, but none or few from non-target tissue libraries. Programmes to assist in the assembly of such sets of overlapping data may be developed in-house or obtained privately or from the internet. For example, the Institute for Genomic Research (TIGR, found at <http://www.tigr.org>) provides many software tools free of charge to the scientific community. Included amongst these is the TIGR assembler (Sutton *et al.* 1995), a tool for the assembly of large sets of overlapping data such as ESTs, bacterial artificial chromosomes (BAC)s, or small genomes. Candidate EST clones representing different genes are then analysed using RNA blot methods for size and tissue specificity and, if required, used as probes to isolate and identify the full length cDNA clone for further characterization. In practice however, the method is rather more involved, requiring bioinformatic and computer analysis coupled with confirmatory molecular studies. Vasmataz *et al.* (1998) have described several problems in this fledgling approach, such as separating highly homologous sequences derived from different genes and an overemphasis of specificity for some EST sequences. However, since these problems will largely be addressed by the development of more suitable computer algorithms and an increased completeness of the EST database, it is likely that this approach to identifying differentially expressed genes may enjoy more patronage in the future.

### Problems and potential of differential expression techniques

#### *The holistic or single cell approach?*

When working with *in vivo* models of differential expression, one of the first issues to consider must be the presence of multiple cell types in any given specimen. For example, a liver sample is likely to contain not only hepatocytes, but also (potentially) Ito cells, bile ductule cells, endothelial cells, various immune cells (e.g. lymphocytes, macrophages and Kupffer cells) and fibroblasts. Other tissues will each have their own distinctive cell populations. Also, in the case of neoplastic tissue, there are almost always normal, hyperplastic and/or dysplastic cells present in a sample. One must, therefore, be aware that genes obtained from a differential display experiment performed on an animal tissue model may not necessarily arise exclusively from the intended 'target' cells, e.g. hepatocytes/neoplastic cells. If appropriate, further analyses using immunohistochemistry, *in situ* hybridization or *in situ* RT-PCR should be used to confirm which cell types are expressing the gene(s) of interest. This problem is probably most acute for those studying the differential expression of genes in the development of different cell types, where there is a need to examine homologous cell populations. The problem is now being addressed at the National Cancer Institute (Bethesda, MD, USA) where new microdissection techniques have been employed to assist in their gene analysis programme, the Cancer Genome Anatomy Project (CGAP) (For more information see web site: <http://www.ncbi.nlm.nih.gov/ncicgap/intro.html>). There are also separation techniques available that utilise cell-specific antigens as a means to isolate target cells,

e.g. fluorescence activated cell sorting (FACS) (Dunbar *et al.* 1998, Kas-Deelen *et al.* 1998) and magnetic bead technology (Richard *et al.* 1998, Rogler *et al.* 1998).

However, those taking a holistic approach may consider this issue unimportant. There is an equally appropriate view that all those genes showing altered expression within a compromised tissue should be taken into consideration. After all, since all tissues are complex mixes of different, interacting cell types which intimately regulate each other's growth and development, it is clear that each cell type could in some way contribute (positively or negatively) towards the molecular mechanisms which lie behind responses to external stimuli or neoplastic growth. It is perhaps then more informative to carry out differential display experiments using *in vivo* as opposed to *in vitro* models, where uniform populations of identical cells probably represent a partial, skewed or even inaccurate picture of the molecular changes that occur.

The incidence and possible implications of inter-individual biological variation should be considered in any approach where whole animal models are being used. It is clear that individuals (humans and animals) respond in different ways to identical stimuli. One of the best characterized examples is the debrisoquine oxidation polymorphism, which is mediated by cytochrome CYP2D6 and determines the pharmacokinetics of many commonly prescribed drugs (Lennard 1993, Meyer and Zanger 1997). The reasons for such differences are varied and complex, but allelic variations, regulatory region polymorphisms and even physical and mental health can all contribute to observed differences in individual responses. Careful thought should, therefore, be given to the specific objectives of the study and to the possible value of pooling starting material (tissue/mRNA). The effect of this can be beneficial through the ironing out of exaggerated responses and unimportant minor fluctuations of (mechanistically) irrelevant genes in individual animals, thus providing a clearer overall picture of the general molecular mechanisms of the response. However, at the same time such minor variations may be of utmost importance in deciding the ability of individual animals to succumb to or resist the effects of a given chemical/disease.

*How efficient are differential expression techniques at recovering a high percentage of differentially expressed genes?*

A number of groups have produced experimental data suggesting that mammalian cells produce between 8000–15 000 different mRNA species at any one time (Mechler and Rabbitts 1981, Hedrick *et al.* 1984, Bravo 1990), although figures as high as 20–30 000 have also been quoted (Axel *et al.* 1976). Hedrick *et al.* (1984) provided evidence suggesting that the majority of these belong to the rare abundance class. A breakdown of this abundance distribution is shown in table 1.

When the results of differential display experiments have been compared with data obtained previously using other methods, it is apparent that not all differentially expressed mRNAs are represented in the final display. In particular, rare messages (which, importantly, often include regulatory proteins) are not easily recovered using differential display systems. This is a major shortcoming, as the majority of mRNA species exist at levels of less than 0.005% of the total population (table 1). Bertoli *et al.* (1995) examined the efficiency of DD templates (heterogeneous mRNA populations) for recovering rare messages and were unable to detect mRNA

species present at less than 1.2% of the total mRNA population—equivalent to an intermediate or abundant species. Interestingly, when simple model systems (single target only) were used instead of a heterogeneous mRNA population, the same primers could detect levels of target mRNA down to 10 000× smaller. These results are probably best explained by competition for substrates from the many PCR products produced in a DD reaction.

The numbers of differentially expressed mRNAs reported in the literature using various model systems provides further evidence that many differentially expressed mRNAs are not recovered. For example, DeRisi *et al.* (1997) used DNA array technology to examine gene expression in yeast following exhaustion of sugar in the medium, and found that more than 1700 genes showed a change in expression of at least 2-fold. In light of such a finding, it would not be unreasonable to suggest that of the 8000–15 000 different mRNA species produced by any given mammalian cell, up to 1000 or more may show altered expression following chemical stimulation. Whilst this may be an extreme figure, it is known that at least 100 genes are activated/upregulated in Jurkat (T-) cells following IL-2 stimulation (Ullman *et al.* 1990). In addition, Wan *et al.* (1996) estimated that interferon- $\gamma$ -stimulated HeLa cells differentially express up to 433 genes (assuming 24 000 distinct mRNAs expressed by the cells). However, there have been few publications documenting anywhere near the recovery of these numbers. For example, in using DD to compare normal and regenerating mouse liver, Bauer *et al.* (1993) found only 70 of 38 000 total bands to be different. Of these, 50% (35 genes) were shown to correspond to differentially expressed bands. Chen *et al.* (1996) reported 10 genes upregulated in female rat liver following ethinyl estradiol treatment. McKenzie and Drake (1997) identified 14 different gene products whose expression was altered by phorbol myristate acetate (PMA, a tumour promoter agent) stimulation of a human myelomonocytic cell line. Kilty and Vickers (1997) identified 10 different gene products whose expression was upregulated in the peripheral blood leukocytes of allergic disease sufferers. Linskens *et al.* (1995) found 23 genes differentially expressed between young and senescent fibroblasts. Techniques other than DD have also provided an apparent paucity of differentially expressed genes. Using SH for example, Cao *et al.* (1997) found 15 genes differentially expressed in colorectal cancer compared to normal mucosal epithelium. Fitzpatrick *et al.* (1995) isolated 17 genes upregulated in rat liver following treatment with the peroxisome proliferator, clofibrate; Philips *et al.* (1990) isolated 12 cDNA clones which were upregulated in highly metastatic mammary adenocarcinoma cell lines compared to poorly metastatic ones. Prashar and Weissman (1996) used 3' restriction fragment analysis and identified approximately 40 genes showing altered expression within 4 h of activation of Jurkat T-cells. Groenink and Leegwater (1996) analysed 27 gene fragments isolated using SSH of delayed early response phase of liver regeneration and found only 12 to be upregulated.

In the laboratory, SSH was used to isolate up to 70 candidate genes which appear to show altered expression in guinea pig liver following short-term treatment with the peroxisome proliferator, WY-14,643 (Rockett, Swales, Esdaile and Gibson, unpublished observations). However, these findings have still to be confirmed by analysis of the extracted tissue mRNA for differential expression of these sequences.

Whilst the latest differential display technologies are purported to include design and experimental modifications to overcome this lack of efficiency (in both the total number of differentially expressed genes recovered and the percentage that are true

positives), it is still not clear if such adaptations are practically effective—proving efficiency by spiking with a known amount of limited numbers of artificial construct(s) is one thing, but isolating a high percentage of the rare messages already present in an mRNA population is another. Of course, some models will genuinely produce only a small number of differentially expressed genes. In addition, there are also technical problems that can reduce efficiency. For example, mRNAs may have an unusual primary structure that effectively prevents their amplification by PCR-based systems. In addition, it is known that under certain circumstances not all mRNAs have 3' polyA sites. For example, during *Xenopus* development, deadenylation is used as a means to stabilize RNAs (Voeltz and Steitz 1998), whilst preferential deadenylation may play a role in regulating Hsp70 (and perhaps, therefore, other stress protein) expression in *Drosophila* (Dellavalle *et al.* 1994). The presence of deadenylated mRNAs would clearly reduce the efficiency of systems utilizing a polydT reverse transcription step. The efficiency of any system also depends on the quality of the starting material. All differential display techniques use mRNA as their target material. However, it is difficult to isolate mRNA that is completely free of ribosomal RNA. Even if polydT primers are used to prime first strand cDNA synthesis, ribosomal RNA is often transcribed to some degree (Clontech PCR-Select cDNA Subtraction kit user manual). It has been shown, at least in the case of SSH, that a high rRNA:mRNA ratio can lead to inefficient subtractive hybridization (Clontech PCR-Select cDNA Subtraction kit user manual), and there is no reason to suppose that it will not do likewise in other SH approaches. Finally, those techniques that utilise a presubtraction amplification step (e.g. RDA) may present a skewed representation since some sequences amplify better than others.

Of course, probably the most important consideration is the temporal factor. It is clear that any given differential display experiment can only interrogate a cell at one point in time. It may well be that a high percentage of the genes showing altered expression at that time are obtained. However, given that disease processes and responses to environmental stimuli involve dynamic cascades of signalling, regulation, production and action, it is clear that all those genes which are switched on/off at different times will not be recovered and, therefore, vital information may well be missed. It is, therefore, imperative to obtain as much information about the model system beforehand as possible, from which a strategy can be derived for targeting specific time points or events that are of particular interest to the investigator. One way of getting round this problem of single time point analysis is to conduct the experiment over a suitable time course which, of course, adds substantially to the amount of work involved.

#### *How sensitive are differential expression technologies?*

There has been little published data that addresses the issue of how large the change in expression must be for it to permit isolation of the gene in question with the various differential expression technologies. Although the isolation of genes whose expression is changed as little as 1.5-fold has been reported using SSH (Groenink and Leegwater 1996), it appears that those demonstrating a change in excess of 5-fold are more likely to be picked up. Thus, there is a 'grey zone' in between where small changes could fade in and out of isolation between



experiments and animals. DD, on the other hand, is not subject to this grey zone since, unlike SH approaches, it does not amplify the difference in expression between two samples. Wan *et al.* (1996) reported that differences in expression of twofold or more are detectable using DD.

#### *Resolution and visualization of differential expression products*

It seems highly improbable with current technology that a gel system could be developed that is able to resolve all gene species showing altered expression in any given test system (be it SH- or DD-based). Polyacrylamide gel electrophoresis (PAGE) can resolve size differences down to 0.2% (Sambrook *et al.* 1989) and are used as standard in DD experiments. Even so, it is clear that a complex series of gene products such as those seen in a DD will contain unresolvable components. Thus, what appears to be one band in a gel may in fact turn out to be several. Indeed, it has been well documented (Mathieu-Daude *et al.* 1996, Smith *et al.* 1997) that a single band extracted from a DD often represents a composite of heterogeneous products, and the same has been found for SSH displays in this laboratory (Rockett *et al.* 1997). One possible solution was offered by Mathieu-Daude *et al.* (1996), who extracted and reamplified candidate bands from a DD display and used single strand conformation polymorphism (SSCP) analysis to confirm which components represented the truly differentially expressed product.

Many scientists often try to avoid the use of PAGE where possible because it is technically more demanding than agarose gel electrophoresis (AGE). Unfortunately, high resolution agarose gels such as Metaphor (FMC, Lichfield, UK) and AquaPor HR (National Diagnostics, Hesse, UK), whilst easier to prepare and manipulate than PAGE, can only separate DNA sequences which differ in size by around 1.5–2% (15–20 base pairs for a 1Kb fragment). Thus, SSH, RDA or other such products which differ in size by less than this amount are normally not resolvable. However, a simple technique does in fact exist for increasing the resolving power of AGE—the inclusion of HA-red (10-phenyl neutral red-PEG ligand) or HA-yellow (bisbenzamide-PEG ligand) (Hanse Analytik GmbH, Bremen, Germany) in a gel separates identical or closely sized products on base content. Specifically, HA-red and -yellow selectively bind to GC and AT DNA motifs, respectively (Wawer *et al.* 1995, Hanse Analytik 1997, personal communication). Since both HA-stains possess an overall positive charge, they migrate towards the cathode when an electric field is applied. This is in direct opposition to DNA, which is negatively charged and, therefore, migrates towards the anode. Thus, if two DNA clones are identical in size (as perceived on a standard high resolution agarose gel), but differ in AT/GC content, inclusion of a HA-dye in the gel will effectively retard the migration of one of the sequences compared to the other, effectively making it apparently larger and, thus, providing a means of differentiating between the two. The use of HA-red has been shown to resolve sequences with an AT variation of less than 1% (Wawer *et al.* 1995), whilst Hanse Analytik have reported that HA staining is so sensitive that in one case it was used to distinguish two 567bp sequences which differed by only a single point mutation (Hanse Analytik 1996, personal communication). Therefore, if one wishes to check whether all the clones produced from a specific band in a differential display experiment are derived from the same gene species, a small amount of reamplified or digested clone can be run on a standard high resolution gel, and a second aliquot

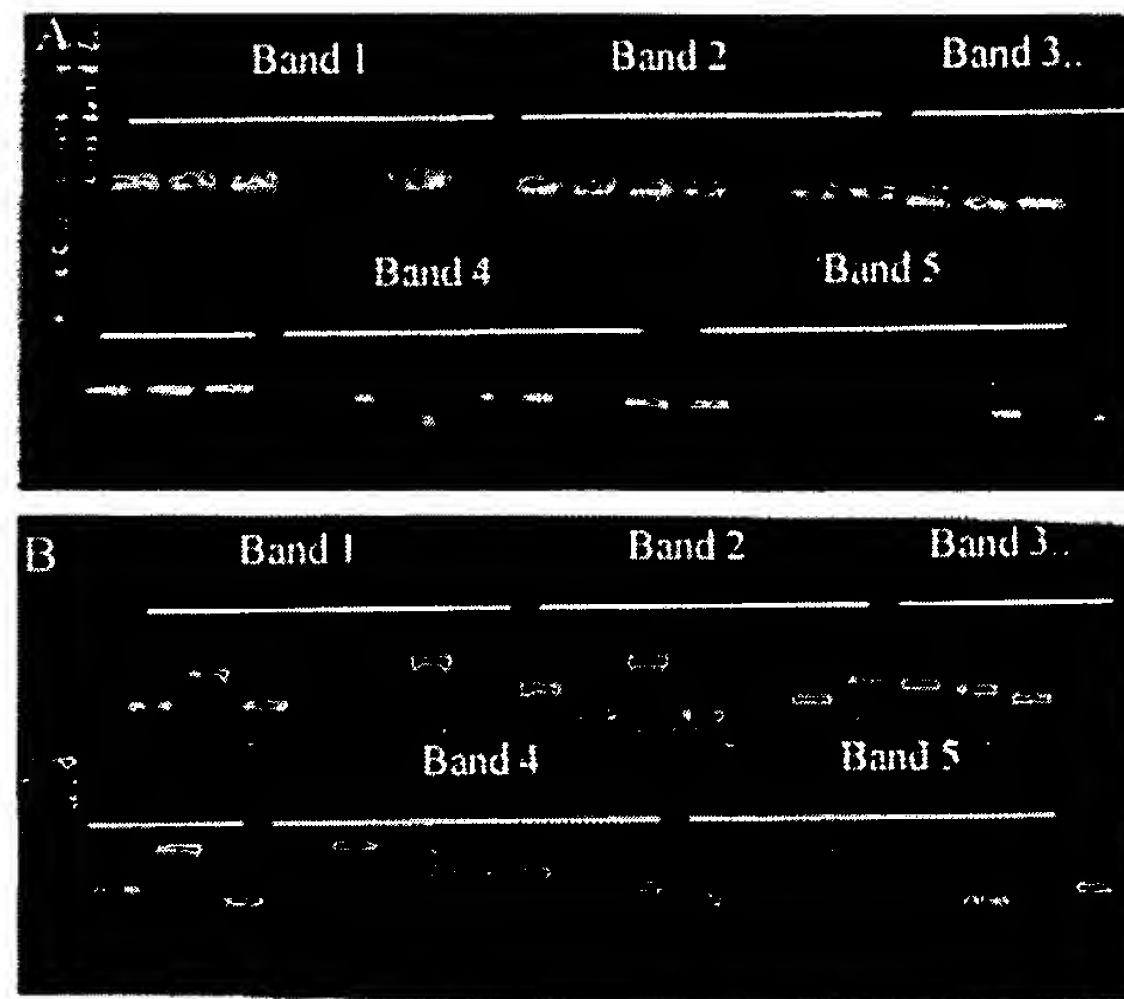


Figure 10. Discrimination of clones of identical/nearly identical size using HA-red. Bands of decreasing size (1–5) were extracted from the final display of a suppression subtractive hybridization experiment and cloned. Seven colonies were picked at random from each cloned band and their inserts amplified using PCR. The products were run on two gels, (A) a high resolution 2% agarose gel, and (B) a high resolution 2% agarose gel containing 1 U/ml HA-red. With few exceptions, all the clones from each band appear to be the same size (gel A). However, the presence of HA-red (gel B), which separates identically-sized DNA fragments based on the percentage of GC within the sequence, clearly indicates the presence of different gene species within each band. For example, even though all five re-amplified clones of band 1 appear to be the same size, at least four different gene species are represented.

in a similar gel containing one of the HA-stains. The standard gel should indicate any gross size differences, whilst the HA-stained gel should separate otherwise unresolvable species (on standard AGE) according to their base content. Geisinger *et al.* (1997) reported successful use of this approach for identifying DD-derived clones. Figure 10 shows such an experiment carried out in this laboratory on clones obtained from a band extracted from an SSH display.

An alternative approach is to carry out a 2-D analysis of the differential display products. In this approach, size-based separation is first carried out in a standard agarose gel. The gel slice containing the display is then extracted and incorporated in to a HA gel for resolution based on AT/GC content.

Of course, one should always consider the possibility of there being different gene species which are the same size and have the same GC/AT content. However, even these species are not unresolvable given some effort—again, one might use SSCP, or perhaps a denaturing gradient gel electrophoresis (DGGE) or temperature gradient field electrophoresis (TGGE) approach to resolve the contents of a band, either directly on the extracted band (Suzuki *et al.* 1991) or on the reamplified product.

The requirement of some differential display techniques to visualize large numbers of products (e.g. DD and GEF) can also present a problem in that, in terms of numbers, the resolution of PAGE rarely exceeds 300–400 bands. One approach to overcoming this might be to use 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991).

Extraction of differentially expressed bands from a gel can be complex since, in some cases (e.g. DD, GEF), the results are visualized by autoradiographic means, such that precise overlay of the developed film on the gel must occur if the correct band is to be extracted for further analysis. Clearly, a misjudged extraction can account for many man-hours lost. This problem, and that of the use of radioisotopes, has been addressed by several groups. For example, Lohmann *et al.* (1995) demonstrated that silver staining can be used directly to visualize DD bands in horizontal PAGs. An *et al.* (1996) avoided the use of radioisotopes by transferring a small amount (20–30%) of the DNA from their DD to a nylon membrane, and visualizing the bands using chemiluminescent staining before going back to extract the remaining DNA from the gel. Chen and Peck (1996) went one step further and transferred the entire DD to a nylon membrane. The DNA bands were then visualized using a digoxigenin (DIG) system (DIG was attached to the polydT primers used in the differential display procedure). Differentially expressed bands were cut from the membrane and the DNA eluted by washing with PCR buffer prior to reamplification.

One of the advantages of using techniques such as SSH and RDA is that the final display can be run on an agarose gel and the bands visualized with simple ethidium bromide staining. Whilst this approach can provide acceptable results, overstaining with SYBR Green I or SYBR Gold nucleic acid stains (FMC) effectively enhances the intensity and sharpness of the bands. This greatly aids in their precise extraction and often reveals some faint products that may otherwise be overlooked. Whilst differential displays stained with SYBR Green I are better visualized using short wavelength UV (254 nm) rather than medium wavelength (306 nm), the shorter wavelength is much more DNA damaging. In practice, it takes only a few seconds to damage DNA extracted under 254 nm irradiation, effectively preventing reamplification and cloning. The best approach is to over stain with SYBR Green I and extract bands under a medium wavelength UV transillumination.

### **The possible use of 'microfingerprinting' to reduce complexity**

Given the sheer number of gene products and the possible complexity of each band, an alternative approach to rapid characterization may be to use an enhanced analysis of a small section of a differential display—a 'sub-fingerprint' or 'micro-fingerprint'. In this case, one could concentrate on those bands which only appear in a particular chosen size region. Reducing the fingerprint in this way has at least two advantages. One is that it should be possible to use different gel types, concentrations and run times tailored exactly to that region. Currently, one might run products from 100–3000 + bp on the same gel, which leads to compromise in the gel system being used and consequently to suboptimal resolution, both in terms of size and numbers, and can lead to problems in the accurate excision of individual bands. Secondly, it may be possible to enhance resolution by using a 2-D analysis using a HA-stain, as described earlier. In summary, if a range of gene product sizes is carefully chosen to include certain 'relevant' genes, the 2-D system standardized, and appropriate gene analysis used, it may be possible to develop a method for the early and rapid identification of compounds which have similar or widely different cellular effects. If the prognosis for exposure to one or more other chemicals which display a similar profile is already known, then one could perhaps predict similar effects for any new compounds which show a similar micro-fingerprint.

An alternative approach to microfingerprinting is to examine altered expression in specific families of genes through careful selection of PCR primers and/or post-reaction analysis. Stress genes, growth factors and/or their receptors, cell cycling genes, cytochromes P450 and regulatory proteins might be considered as candidates for analysis in this way. Indeed, some off-the-shelf DNA arrays (e.g. Clontech's Atlas cDNA Expression Array series) already anticipated this to some degree by grouping together genes involved in different responses e.g. apoptosis, stress, DNA-damage response etc.

## Screening

### *False positives*

The generation of false positives has been discussed at length amongst the differential display community (Liang *et al.* 1993, 1995, Nishio *et al.* 1994, Sun *et al.* 1994, Sompayrac *et al.* 1995). The reason for false positives varies with the technique being used. For instance, in RDA, the use of adaptors which have not been HPLC purified can lead to the production of false positives through illegitimate ligation events (O'Neill and Sinclair 1997), whilst in DD they can arise through PCR artifacts and illegitimate transcription of rRNA. In SH, false positives appear to be derived largely from abundant gene species, although some may arise from cDNA/mRNA species which do not undergo hybridization for technical reasons.

A quick screening of putative differentially expressed clones can be carried out using a simple dot blot approach, in which labelled first strand probes synthesized from tester and driver mRNA are hybridized to an array of said clones (Hedrick *et al.* 1984, Sakaguchi *et al.* 1986). Differentially expressed clones will hybridize to tester probe, but not driver. The disadvantage of this approach is that rare species may not generate detectable hybridization signals. One option for those using SSH is to screen the clones using a labelled probe generated from the subtracted cDNA from which it was derived, and with a probe made from the reverse subtraction reaction (ClonTechniques 1997a). Since the SSH method enriches rare sequences, it should be possible to confirm the presence of clones representing low abundance genes. Despite this quick screening step, there is still the need to go back to the original mRNA and confirm the altered expression using a more quantitative approach. Although this may be achieved using Northern blots, the sensitivity is poor by today's high standards and one must rely on PCR methods for accurate and sensitive determinations (see below).

## Sequence analysis

The majority of differential display procedures produce final products which are between 100 and 1000bp in size. However, this may considerably reduce the size of the sequence for analysis of the DNA databases. This in turn leads to a reduced confidence in the result—several families of genes have members whose DNA sequences are almost identical except in a few key stretches, e.g. the cytochrome P450 gene superfamily (Nelson *et al.* 1996). Thus, does the clone identified as being almost identical to gene  $X_0$  really come from that gene, or its brother gene  $X_1$  or its as yet undiscovered sister  $X_2$ ? For example, using SSH, part of a gene was isolated,



which was up-regulated in the liver of rats exposed to Wy-14,643 and was identified by a FASTA search as being transferrin (data not shown). However, transferrin is known to be downregulated by hypolipidemic peroxisome proliferators such as Wy-14,643 (Hertz *et al.* 1996), and this was confirmed with subsequent RT-PCR analysis. This suggests that the gene sequence isolated may belong to a gene which is closely related to transferrin, but is regulated by a different mechanism.

A further problem associated with SH technology is redundancy. In most cases before SH is carried out, the cDNA population must first be simplified by restriction digestion. This is important for at least two reasons:

- (1) To reduce complexity—long cDNA fragments may form complex networks which prevent the formation of appropriate hybrids, especially at the high concentrations required for efficient hybridization.
- (2) Cutting the cDNAs into small fragments provides better representation of individual genes. This is because genes derived from related but distinct members of gene families often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (Ko 1990). Furthermore, different fragments from the same cDNA may differ considerably in terms of hybridization and amplification and, thus, may not efficiently do one or the other (Wang and Brown 1991). Thus, some fragments from differentially expressed cDNAs may be eliminated during subtractive hybridization procedures. However, other fragments may be enriched and isolated. As a consequence of this, some genes will be cut one or more times, giving rise to two or more fragments of different sizes. If those same genes are differentially expressed, then two or more of the different size fragments may come through as separate bands on the final differential display, increasing the observed redundancy and increasing the number of redundant sequencing reactions.

Sequence comparisons also throw up another important point—at what degree of sequence similarity does one accept a result. Is 90% identity between a gene derived from your model species and another acceptably close? Is 95% between your sequence and one from the same species also acceptable? This problem is particularly relevant when the forward and reverse sequence comparisons give similar sequences with completely different gene species! An arbitrary decision seems to be to allocate genes that are definite (95% and above similarity) and then group those between 60 and 95% as being related or possible homologues.

### Quantitative analysis

At some point, one must give consideration to the quantitative analysis of the candidate genes, either as a means of confirming that they are truly differentially expressed, or in order to establish just what the differences are. Northern blot analysis is a popular approach as it is relatively easy and quick to perform. However, the major drawback with Northern blots is that they are often not sensitive enough to detect rare sequences. Since the majority of messages expressed in a cell are of low abundance (see table 1), this is a major problem. Consequently, RT-PCR may be the method of choice for confirming differential expression. Although the procedure is somewhat more complex than Northern analysis, requiring synthesis of primers and optimization of reaction conditions for each gene species, it is now possible to set up high throughput PCR systems using multichannel pipettes, 96 +-well plates and

appropriate thermal cycling technology. Whilst quantitative analysis is more desirable, being more accurate and without reliance on an internal standard, the money and time needed to develop a competitor molecule is often excessive, especially when one might be examining tens or even hundreds of gene species. The use of semi-quantitative analysis is simpler, although still relatively involved. One must first of all choose an internal standard that does not change in the test cells compared to the controls. Numerous reference genes have been tried in the past, for example interferon-gamma (IFN- $\gamma$ , Frye *et al.* 1989),  $\beta$ -actin (Heuval *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Wong *et al.* 1994), dihydrofolate reductase (DHFR, Mohler and Butler 1991),  $\beta$ -2-microglobulin ( $\beta$ -2-m, Murphy *et al.* 1990), hypoxanthine phosphoribosyl transferase (HPRT, Foss *et al.* 1998) and a number of others (ClonTechniques 1997b). Ideally, an internal standard should not change its level of expression in the cell regardless of cell age, stage in the cell cycle or through the effects of external stimuli. However, it has been shown on numerous occasions that the levels of most housekeeping genes currently used by the research community do in fact change under certain conditions and in different tissues (ClonTechniques 1997b). It is imperative, therefore, that preliminary experiments be carried out on a panel of housekeeping genes to establish their suitability for use in the model system.

Interpretation of quantitative data must also be treated with caution. By comparing the lists of genes identified by differential expression one can perhaps gain insight into why two different species react in different ways to external stimuli. For example, rats and mice appear sensitive to the non-genotoxic effects of a wide range of peroxisome proliferators whilst Syrian hamsters and guinea pigs are largely resistant (Orton *et al.* 1984, Rodricks and Turnbull 1987, Lake *et al.* 1989, 1993, Makowska *et al.* 1992). A simplified approach to resolving the reason(s) why is to compare lists of up- and down-regulated genes in order to identify those which are expressed in only one species and, through background knowledge of the effects of the said gene, might suggest a mechanism of facilitated non-genotoxic carcinogenesis or protection. Of course, the situation is likely to be far more complex. Perhaps if there were one key gene protecting guinea pig from non-genotoxic effects and it was upregulated 50 times by PPs, the same gene might only be up-regulated five times in the rat. However, since both were noted to be upregulated, the importance of the gene may be overlooked. Just to complicate matters, a large change in expression does not necessarily mean a biologically important change. For example, what is the true relevance of gene Y which shows a 50-fold increase after a particular treatment, and gene Z which shows only a 5-fold increase? If one examines the literature one may find that historically, gene Y has often been shown to be up-regulated 40–60-fold by a number of unrelated stimuli—in light of this the 50-fold increase would appear less significant. However, the literature may show that gene Z has never been recorded as having more than doubled in expression—which makes your 5-fold increase all the more exciting. Perhaps even more interesting is if that same 5-fold increase has only been seen in related neoplasms or following treatment with related chemicals.

#### **Problems in using the differential display approach**

Differential display technology originally held promise of an easily obtainable 'fingerprint' of those genes which are up- or down-regulated in test animals/cells in a developmental process or following exposure to given stimuli. However, it has

become clear that the fingerprinting process, whilst still valid, is much too complex to be represented by a single technique profile. This is because all differential display techniques have common and/or unique technical problems which preclude the isolation and identification of all those genes which show changes in expression. Furthermore, there are important genetic changes related to disease development which differential expression analysis is simply not designed to address. An example of this is the presence of small deletions, insertions, or point mutations such as those seen in activated oncogenes, tumour suppressor genes and individual polymorphisms. Polymorphic variations, small though they usually are, are often regarded as being of paramount importance in explaining why some patients respond better than others to certain drug treatments (and, in logical extension, why some people are less affected by potentially dangerous xenobiotics/carcinogens than others). The identification of such point mutations and naturally occurring polymorphisms requires the subsequent application of sequencing, SSCP, DGGE or TGGE to the gene of interest. Furthermore, differential display is not designed to address issues such as alternatively spliced gene species or whether an increased abundance of mRNA is a result of increased transcription or increased mRNA stability.

### *Conclusions*

Perhaps the main advantage of open system differential display techniques is that they are not limited by extant theories or researcher bias in revealing genes which are differentially expressed, since they are designed to amplify all genes which demonstrate altered expression. This means that they are useful for the isolation of previously unknown genes which may turn out be useful biomarkers of a particular state or condition. At least one open system (SAGE) is also quantitative, thus eliminating the need to return to the original mRNA and carry out Northern/PCR analysis to confirm the result. However, the rapid progress of genome mapping projects means that over the next 5–10 years or so, the balance of experimental use will switch from open to closed differential display systems, particularly DNA arrays. Arrays are easier and faster to prepare and use, provide quantitative data, are suitable for high throughput analysis and can be tailored to look at specific signalling pathways or families of genes. Identification of all the gene sequences in human and common laboratory animals combined with improved DNA array technology, means that it will soon no longer be necessary to try to isolate differentially expressed genes using the technically more demanding open system approach. Thus, their main advantage (that of identifying unknown genes) will be largely eradicated. It is likely, therefore, that their sphere of application will be reduced to analysis of the less common laboratory species, since it will be some time yet before the genomes of such animals as zebrafish, electric eels, gerbils, crayfish and squid, for example, will be sequenced.

Of course, in the end the question will always remain: What is the functional/biological significance of the identified, differentially expressed genes? One persistent problem is understanding whether differentially expressed genes are a cause or consequence of the altered state. Furthermore, many chemicals, such as non-genotoxic carcinogens, are also mitogens and so genes associated with replication will also be upregulated but may have little or nothing to do with the

carcinogenic effect. Whilst differential display technology cannot hope to answer these questions, it does provide a springboard from which identification, regulatory and functional studies can be launched. Understanding the molecular mechanism of cellular responses is almost impossible without knowing the regulation and function of those genes and their condition (e.g. mutated). In an abstract sense, differential display can be likened to a still photograph, showing details of a fixed moment in time. Consider the Historian who knows the outcome of a battle and the placement and condition of the troops before the battle commenced, but is asked to try and deduce how the battle progressed and why it ended as it did from a few still photographs—an impossible task. In order to understand the battle, the Historian must find out the capabilities and motivation of the soldiers and their commanding officers, what the orders were and whether they were obeyed. He must examine the terrain, the remains of the battle and consider the effects the prevailing weather conditions exerted. Likewise, if mechanistic answers are to be forthcoming, the scientist must use differential display in combination with other techniques, such as knockout technology, the analysis of cell signalling pathways, mutation analysis and time and dose response analyses. Although this review has emphasized the importance of differential gene profiling, it should not be considered in isolation and the full impact of this approach will be strengthened if used in combination with functional genomics and proteomics (2-dimensional protein gels from isoelectric focusing and subsequent SDS electrophoresis and virtual 2D-maps using capillary electrophoresis). Proteomics is attracting much recent attention as many of the changes resulting in differential gene expression do not involve changes in mRNA levels, as described extensively herein, but rather protein–protein, protein–DNA and protein phosphorylation events which would require functional genomics or proteomic technologies for investigation.

Despite the limitations of differential display technology, it is clear that many potential applications and benefits can be obtained from characterizing the genetic changes that occur in a cell during normal and disease development and in response to chemical or biological insult. In light of functional data, such profiling will provide a ‘fingerprint’ of each stage of development or response, and in the long term should help in the elucidation of specific and sensitive biomarkers for different types of chemical/biological exposure and disease states. The potential medical and therapeutic benefits of understanding such molecular changes are almost immeasurable. Amongst other things, such fingerprints could indicate the family or even specific type of chemical an individual has been exposed to plus the length and/or acuteness of that exposure, thus indicating the most prudent treatment. They may also help uncover differences in histologically identical cancers, provide diagnostic tests for the earliest stages of neoplasia and, again, perhaps indicate the most efficacious treatment.

The Human Genome Project will be completed early in the next century and the DNA sequence of all the human genes will be known. The continuing development and evolution of differential gene expression technology will ensure that this knowledge contributes fully to the understanding of human disease processes.

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# Microarrays and Toxicology: The Advent of Toxicogenomics

Emile F. Nuwaysir,<sup>1</sup> Michael Bittner,<sup>2</sup> Jeffrey Trent,<sup>2</sup> J. Carl Barrett,<sup>1</sup> and Cynthia A. Afshari<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

<sup>2</sup>Laboratory of Cancer Genetics, National Human Genome Research Institute, Bethesda, Maryland

The availability of genome-scale DNA sequence information and reagents has radically altered life-science research. This revolution has led to the development of a new scientific subdiscipline derived from a combination of the fields of toxicology and genomics. This subdiscipline, termed toxicogenomics, is concerned with the identification of potential human and environmental toxicants, and their putative mechanisms of action, through the use of genomics resources. One such resource is DNA microarrays or "chips," which allow the monitoring of the expression levels of thousands of genes simultaneously. Here we propose a general method by which gene expression, as measured by cDNA microarrays, can be used as a highly sensitive and informative marker for toxicity. Our purpose is to acquaint the reader with the development and current state of microarray technology and to present our view of the usefulness of microarrays to the field of toxicology. *Mol. Carcinog.* 24:153-159, 1999. © 1999 Wiley-Liss, Inc.

Key words: toxicology; gene expression; animal bioassay

## INTRODUCTION

Technological advancements combined with intensive DNA sequencing efforts have generated an enormous database of sequence information over the past decade. To date, more than 3 million sequences, totaling over 2.2 billion bases [1], are contained within the GenBank database, which includes the complete sequences of 19 different organisms [2]. The first complete sequence of a free-living organism, *Haemophilus influenzae*, was reported in 1995 [3] and was followed shortly thereafter by the first complete sequence of a eukaryote, *Saccharomyces cerevisiae* [4]. The development of dramatically improved sequencing methodologies promises that complete elucidation of the *Homo sapiens* DNA sequence is not far behind [5].

To exploit more fully the wealth of new sequence information, it was necessary to develop novel methods for the high-throughput or parallel monitoring of gene expression. Established methods such as northern blotting, RNase protection assays, S1 nuclease analysis, plaque hybridization, and slot blots do not provide sufficient throughput to effectively utilize the new genomics resources. Newer methods such as differential display [6], high-density filter hybridization [7,8], serial analysis of gene expression [9], and cDNA- and oligonucleotide-based microarray "chip" hybridization [10-12] are possible solutions to this bottleneck. It is our belief that the microarray approach, which allows the monitoring of expression levels of thousands of genes simultaneously, is a tool of unprecedented power for use in toxicology studies.

Almost without exception, gene expression is altered during toxicity, as either a direct or indirect result of toxicant exposure. The challenge facing toxicologists is to define, under a given set of experimental conditions, the characteristic and specific pattern of gene expression elicited by a given toxicant. Microarray technology offers an ideal platform for this type of analysis and could be the foundation for a fundamentally new approach to toxicology testing.

## MICROARRAY DEVELOPMENT AND APPLICATIONS

### cDNA Microarrays

In the past several years, numerous systems were developed for the construction of large-scale DNA arrays. All of these platforms are based on cDNAs or oligonucleotides immobilized to a solid support. In the cDNA approach, cDNA (or genomic) clones of interest are arrayed in a multi-well format and amplified by polymerase chain reaction. The products of this amplification, which are usually 500- to 2000-bp clones from the 3' regions of the genes of interest, are then spotted onto solid support by using high-speed robotics. By using this method, microarrays of up to 10 000 clones can be generated by spotting onto a glass substrate

\*Correspondence to: Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, 111 Alexander Drive, Research Triangle Park, NC 27709.

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; NIEHS, National Institute of Environmental Health Sciences.

[13,14]. Sample detection for microarrays on glass involves the use of probes labeled with fluorescent or radioactive nucleotides.

Fluorescent cDNA probes are generated from control and test RNA samples in single-round reverse-transcription reactions in the presence of fluorescently tagged dUTP (e.g., Cy3-dUTP and Cy5-dUTP), which produces control and test products labeled with different fluorors. The cDNAs generated from these two populations, collectively termed the "probe," are then mixed and hybridized to the array under a glass coverslip [10,11,15]. The fluorescent signal is detected by using a custom-designed scanning confocal microscope equipped with a motorized stage and lasers for fluor excitation [10,11,15]. The data are analyzed with custom digital image analysis software that determines for each DNA feature the ratio of fluor 1 to fluor 2, corrected for local background [16,17]. The strength of this approach lies in the ability to label RNAs from control and treated samples with different fluorescent nucleotides, allowing for the simultaneous hybridization and detection of both populations on one microarray. This method eliminates the need to control for hybridization between arrays. The research groups of Drs. Patrick Brown and Ron Davis at Stanford University spearheaded the effort to develop this approach, which has been successfully applied to studies of *Arabidopsis thaliana* RNA [10], yeast genomic DNA [15], tumorigenic versus non-tumorigenic human tumor cell lines [11], human T-cells [18], yeast RNA [19], and human inflammatory disease-related genes [20]. The most dramatic result of this effort was the first published account of gene expression of an entire genome, that of the yeast *Saccharomyces cerevisiae* [21].

In an alternative approach, large numbers of cDNA clones can be spotted onto a membrane support, albeit at a lower density [7,22]. This method is useful for expression profiling and large-scale screening and mapping of genomic or cDNA clones [7,22-24]. In expression profiling on filter membranes, two different membranes are used simultaneously for control and test RNA hybridizations, or a single membrane is stripped and reprobed. The signal is detected by using radioactive nucleotides and visualized by phosphorimager analysis or autoradiography. Numerous companies now sell such cDNA membranes and software to analyze the image data [25-27].

#### Oligonucleotide Microarrays

Oligonucleotide microarrays are constructed either by spotting prefabricated oligos on a glass support [13] or by the more elegant method of direct in situ oligo synthesis on the glass surface by photolithography [28-30]. The strength of this approach lies in its ability to discriminate DNA molecules based on single base-pair difference. This allows the application of this method to the fields of medical diagnos-

tics, pharmacogenetics, and sequencing by hybridization as well as gene-expression analysis.

Fabrication of oligonucleotide chips by photolithography is theoretically simple but technically complex [29,30]. The light from a high-intensity mercury lamp is directed through a photolithographic mask onto the silica surface, resulting in deprotection of the terminal nucleotides in the illuminated regions. The entire chip is then reacted with the desired free nucleotide, resulting in selected chain elongation. This process requires only  $4n$  cycles (where  $n$  = oligonucleotide length in bases) to synthesize a vast number of unique oligos, the total number of which is limited only by the complexity of the photolithographic mask and the chip size [29,31,32].

Sample preparation involves the generation of double-stranded cDNA from cellular poly(A)<sup>+</sup> RNA followed by antisense RNA synthesis in an in vitro transcription reaction with biotinylated or fluor-tagged nucleotides. The RNA probe is then fragmented to facilitate hybridization. If the indirect visualization method is used, the chips are incubated with fluor-linked streptavidin (e.g., phycoerythrin) after hybridization [12,33]. The signal is detected with a custom confocal scanner [34]. This method has been applied successfully to the mapping of genomic library clones [35], to de novo sequencing by hybridization [28,36], and to evolutionary sequence comparison of the *BRCA1* gene [37]. In addition, mutations in the cystic fibrosis [38] and *BRCA1* [39] gene products and polymorphisms in the human immunodeficiency virus-1 clade B protease gene [40] have been detected by this method. Oligonucleotide chips are also useful for expression monitoring [33] as has been demonstrated by the simultaneous evaluation of gene-expression patterns in nearly all open reading frames of the yeast strain *S. cerevisiae* [12]. More recently, oligonucleotide chips have been used to help identify single nucleotide polymorphisms in the human [41] and yeast [42] genomes.

#### THE USE OF MICROARRAYS IN TOXICOLOGY

##### Screening for Mechanism of Action

The field of toxicology uses numerous in vivo model systems, including the rat, mouse, and rabbit, to assess potential toxicity and these bioassays are the mainstay of toxicology testing. However, in the past several decades, a plethora of in vitro techniques have been developed to measure toxicity, many of which measure toxicant-induced DNA damage. Examples of these assays include the Ames test, the Syrian hamster embryo cell transformation assay, micronucleus assays, measurements of sister chromatid exchange and unscheduled DNA synthesis, and many others. Fundamental to all of these methods is the fact that toxicity is often preceded by, and results in, alterations in gene expression. In many cases, these changes in gene expression are a

far more sensitive, characteristic, and measurable endpoint than the toxicity itself. We therefore propose that a method based on measurements of the genome-wide gene expression pattern of an organism after toxicant exposure is fundamentally informative and complements the established methods described above.

We are developing a method by which toxicants can be identified and their putative mechanisms of action determined by using toxicant-induced gene expression profiles. In this method, in one or more defined model systems, dose and time-course parameters are established for a series of toxicants within a given prototypic class (e.g., polycyclic aromatic hydrocarbons (PAHs)). Cells are then treated with these agents at a fixed toxicity level (as measured by cell survival), RNA is harvested, and toxicant-induced gene expression changes are assessed by hybridization to a cDNA microarray chip (Figure 1). We have developed a custom DNA chip, called ToxChip v1.0, specifically for this purpose and will discuss it in more detail below. The changes in gene expression induced by the test agents in the model systems are analyzed, and the common set of changes unique to that class of toxicants, termed a toxicant signature, is determined.

This signature is derived by ranking across all experiments the gene-expression data based on rela-

tive fold induction or suppression of genes in treated samples versus untreated controls and selecting the most consistently different signals across the sample set. A different signature may be established for each prototypic toxicant class. Once the signatures are determined, gene-expression profiles induced by unknown agents in these same model systems can then be compared with the established signatures. A match assigns a putative mechanism of action to the test compound. Figure 2 illustrates this signature method for different types of oxidant stressors, PAHs, and peroxisome proliferators. In this example, the unknown compound in question had a gene-expression profile similar to that of the oxidant stressors in the database. We anticipate that this general method will also reveal cross talk between different pathways induced by a single agent (e.g., reveal that a compound has both PAH-like and oxidant-like properties). In the future, it may be necessary to distinguish very subtle differences between compounds within a very large sample set (e.g., thousands of highly similar structural isomers in a combinatorial chemistry library or peptide library). To generate these highly refined signatures, standard statistical clustering techniques or principal-component analysis can be used.

For the studies outlined in Figure 2, we developed the custom cDNA microarray chip ToxChip v1.0.

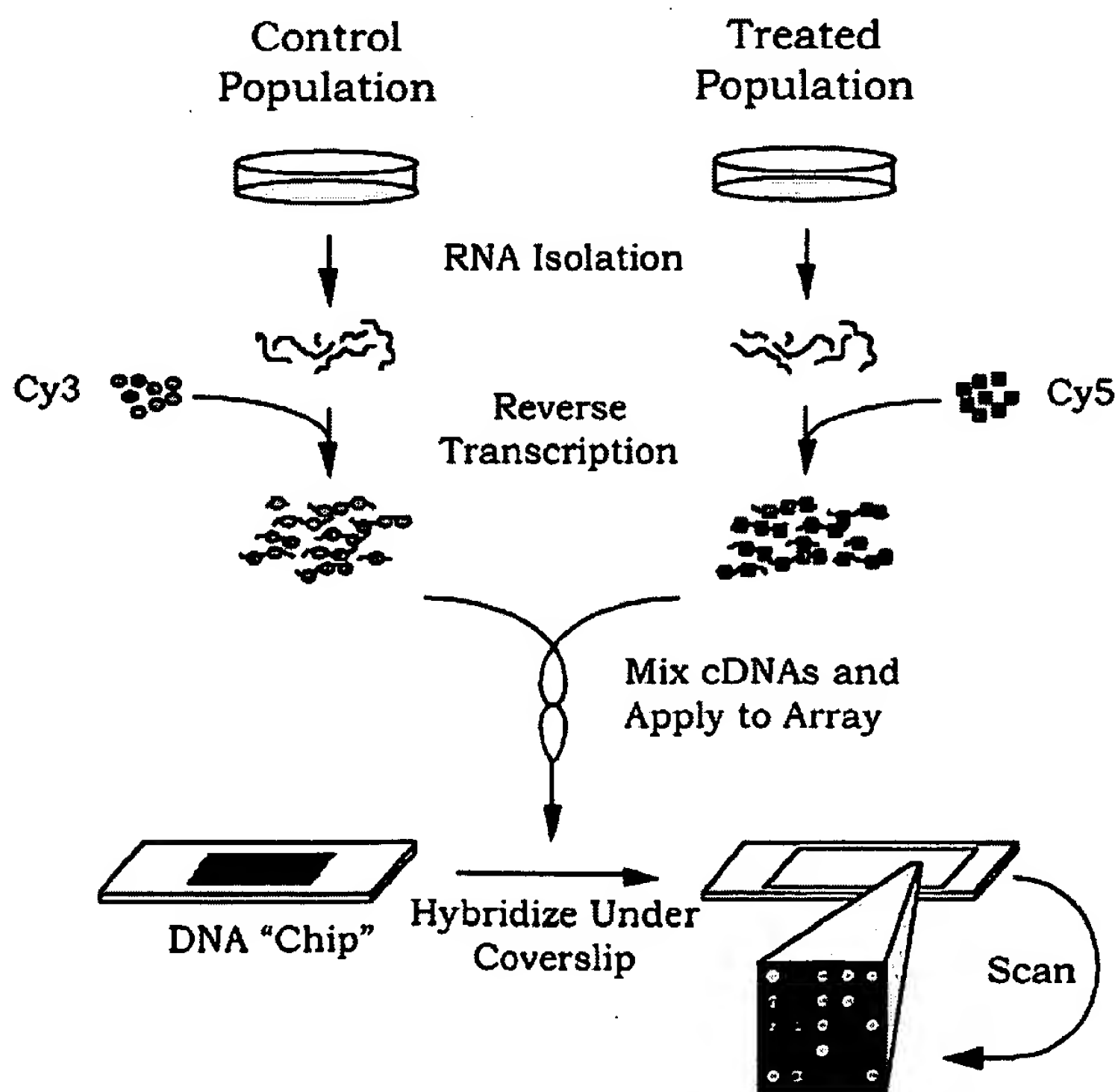


Figure 1. Simplified overview of the method for sample preparation and hybridization to cDNA microarrays. For illus-

trative purposes, samples derived from cell culture are depicted, although other sample types are amenable to this analysis.



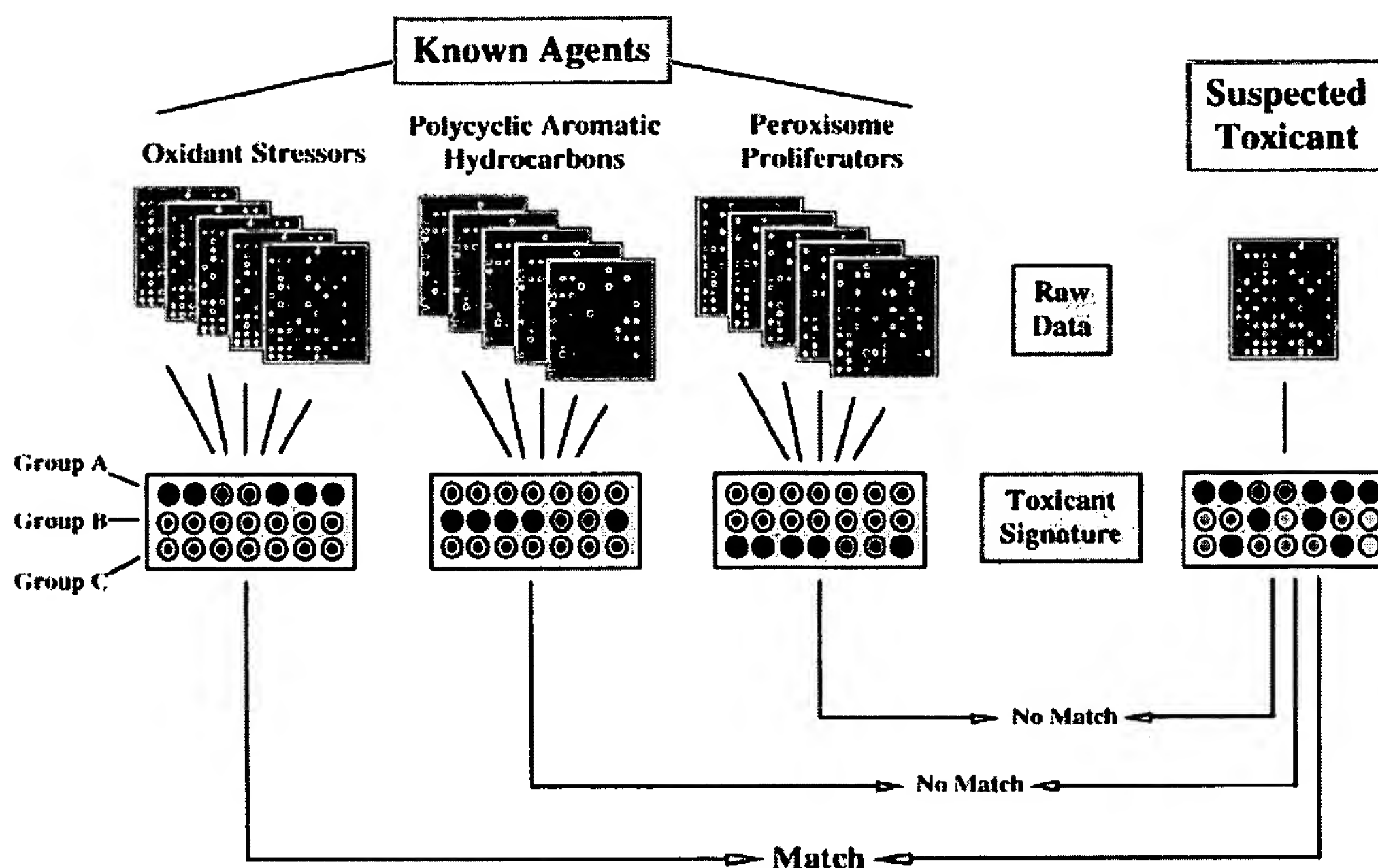


Figure 2. Schematic representation of the method for identification of a toxicant's mechanism of action. In this method, gene-expression data derived from exposure of model systems to known toxicants are analyzed, and a set of changes characteristic to that type of toxicant (termed the toxicant signature) is identified. As depicted, oxidant stressors produce

consistent changes in group A genes (indicated by red and green circles), but not group B or C genes (indicated by gray circles). The set of gene-expression changes elicited by the suspected toxicant is then compared with these characteristic patterns, and a putative mechanism of action is assigned to the unknown agent.

The 2090 human genes that comprise this subarray were selected for their well-documented involvement in basic cellular processes as well as their responses to different types of toxic insult. Included on this list are DNA replication and repair genes, apoptosis genes, and genes responsive to PAHs and dioxin-like compounds, peroxisome proliferators, estrogenic compounds, and oxidant stress. Some of the other categories of genes include transcription factors, oncogenes, tumor suppressor genes, cyclins, kinases, phosphatases, cell adhesion and motility genes, and homeobox genes. Also included in this group are 84 housekeeping genes, whose hybridization intensity is averaged and used for signal normalization of the other genes on the chip. To date, very few toxicants have been shown to have appreciable effects on the expression of these housekeeping genes. However, this housekeeping list will be revised if new data warrant the addition or deletion of a particular gene. Table 1 contains a general description of some of the different classes of genes that comprise ToxChip v1.0.

When a toxicant signature is determined, the genes within this signature are flagged within the database. When uncharacterized toxicants are then screened, the data can be quickly reformatted so that blocks of genes representing the different signatures

are displayed [11]. This facilitates rapid, visual interpretation of data. We are also developing ToxChip v2.0 and chips for other model systems, including rat, mouse, *Xenopus*, and yeast, for use in toxicology studies.

#### Animal Models in Toxicology Testing

The toxicology community relies heavily on the use of animals as model systems for toxicology testing. Unfortunately, these assays are inherently expensive, require large numbers of animals and take a long time to complete and analyze. Therefore, the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program, and the toxicology community at large are committed to reducing the number of animals used, by developing more efficient and alternative testing methodologies. Although substantial progress has been made in the development of alternative methods, bioassays are still used for testing endpoints such as neurotoxicity, immunotoxicity, reproductive and developmental toxicology, and genetic toxicology. The rodent cancer bioassay is a particularly expensive and time-consuming assay, as it requires almost 4 yr, 1200 animals, and millions of dollars to execute and analyze [43]. In vitro experiments of the type outlined in Figure 2 might provide evidence that an unknown

Table 1. ToxChip v1.0: A Human cDNA Microarray Chip Designed to Detect Responses to Toxic Insult

Gene category	No. of genes on chip
Apoptosis	72
DNA replication and repair	99
Oxidative stress/redox homeostasis	90
Peroxisome proliferator responsive	22
Dioxin/PAH responsive	12
Estrogen responsive	63
Housekeeping	84
Oncogenes and tumor suppressor genes	76
Cell-cycle control	51
Transcription factors	131
Kinases	276
Phosphatases	88
Heat-shock proteins	23
Receptors	349
Cytochrome P450s	30

\*This list is intended as a general guide. The gene categories are not unique, and some genes are listed in multiple categories.

agent is (or is not) responsible for eliciting a given biological response. This information would help to select a bioassay more specifically suited to the agent in question or perhaps suggest that a bioassay is not necessary, which would dramatically reduce cost, animal use, and time.

The addition of microarray techniques to standard bioassays may dramatically enhance the sensitivity and interpretability of the bioassay and possibly reduce its cost. Gene-expression signatures could be determined for various types of tissue-specific toxicants, and new compounds could be screened for these characteristic signatures, providing a rapid and sensitive *in vivo* test. Also, because gene expression is often exquisitely sensitive to low doses of a toxicant, the combination of gene-expression screening and the bioassay might allow the use of lower toxicant doses, which are more relevant to human exposure levels, and the use of fewer animals. In addition, gene-expression changes are normally measured in hours or days, not in the months to years required for tumor development. Furthermore, microarrays might be particularly useful for investigating the relationship between acute and chronic toxicity and identifying secondary effects of a given toxicant by studying the relationship between the duration of exposure to a toxicant and the gene-expression profile produced. Thus, a bioassay that incorporates gene-expression signatures with traditional endpoints might be substantially shorter, use more realistic dose regimens, and cost substantially less than the current assays do.

These considerations are also relevant for branches of toxicology not related to human health and not using rodents as model systems, such as aquatic toxicology and plant pathology. Bioassays based on the flathead minnow, *Daphnia*, and *Arabidopsis* could

also be improved by the addition of microarray analysis. The combination of microarrays with traditional bioassays might also be useful for investigating some of the more intractable problems in toxicology research, such as the effects of complex mixtures and the difficulties in cross-species extrapolation.

#### Exposure Assessment, Environmental Monitoring, and Drug Safety

The currently used methods for assessment of exposure to chemical toxicants are based on measurement of tissue toxin levels or on surrogate markers of toxicity, termed biomarkers (e.g., peripheral blood levels of hepatic enzymes or DNA adducts). Because gene expression is a sensitive endpoint, gene expression as measured with microarray technology may be useful as a new biomarker to more precisely identify hazards and to assess exposure. Similarly, microarrays could be used in an environmental-monitoring capacity to measure the effect of potential contaminants on the gene-expression profiles of resident organisms. In an analogous fashion, microarrays could be used to measure gene-expression endpoints in subjects in clinical trials. The combination of these gene-expression data and more established toxic endpoints in these trials could be used to define highly precise surrogates of safety.

Gene-expression profiles in samples from exposed individuals could be compared to the profiles of the same individuals before exposure. From this information, the nature of the toxic exposure can be determined or a relative clinical safety factor estimated. In the future it may also be possible to estimate not only the nature but the dose of the toxicant for a given exposure, based on relative gene-expression levels. This general approach may be particularly appropriate for occupational-health applications, in which unexposed and exposed samples from the same individuals may be obtainable. For example, a pilot study of gene expression in peripheral-blood lymphocytes of Polish coke-oven workers exposed to PAHs (and many other compounds) is under consideration at the NIEHS. An important consideration for these types of studies is that gene expression can be affected by numerous factors, including diet, health, and personal habits. To reduce the effects of these confounding factors, it may be necessary to compare pools of control samples with pools of treated samples. In the future it may be possible to compare exposed sample sets to a national database of human-expression data, thus eliminating the need to provide an unexposed sample from the same individual. Efforts to develop such a national gene-expression database are currently under way [44,45]. However, this national database approach will require a better understanding of genome-wide gene expression across the highly diverse human population and of the effects of environmental factors on this expression.

### Alleles, Oligo Arrays, and Toxicogenetics

Gene sequences vary between individuals, and this variability can be a causative factor in human diseases of environmental origin [46,47]. A new area of toxicology, termed toxicogenetics, was recently developed to study the relationship between genetic variability and toxicant susceptibility. This field is not the subject of this discussion, but it is worthwhile to note that the ability of oligonucleotide arrays to discriminate DNA molecules based on single base-pair differences makes these arrays uniquely useful for this type of analysis. Recent reports demonstrated the feasibility of this approach [41,42]. The NIEHS has initiated the Environmental Genome Project to identify common sequence polymorphisms in 200 genes thought to be involved in environmental diseases [48]. In a pilot study on the feasibility of this application to the Environmental Genome Project, oligonucleotide arrays will be used to resequence 20 candidate genes. This toxicogenetic approach promises to dramatically improve our understanding of interindividual variability in disease susceptibility.

### FUTURE PRIORITIES

There are many issues that must be addressed before the full potential of microarrays in toxicology research can be realized. Among these are model system selection, dose selection, and the temporal nature of gene expression. In other words, in which species, at what dose, and at what time do we look for toxicant-induced gene expression? If human samples are analyzed, how variable is global gene expression between individuals, before and after toxicant exposure? What are the effects of age, diet, and other factors on this expression? Experience, in the form of large data sets of toxicant exposures, will answer these questions.

One of the most pressing issues for array scientists is the construction of a national public database (linked to the existing public databases) to serve as a repository for gene-expression data. This relational database must be made available for public use, and researchers must be encouraged to submit their expression data so that others may view and query the information. Researchers at the National Institutes of Health have made laudable progress in developing the first generation of such a database [44,45]. In addition, improved statistical methods for gene clustering and pattern recognition are needed to analyze the data in such a public database.

The proliferation of different platforms and methods for microarray hybridizations will improve sample handling and data collection and analysis and reduce costs. However, the variety of microarray methods available will create problems of data compatibility between platforms. In addition, the near-infinite variety of experimental conditions under

which data will be collected by different laboratories will make large-scale data analysis extremely difficult. To help circumvent these future problems, a set of standards to be included on all platforms should be established. These standards would facilitate data entry into the national database and serve as reference points for cross-platform and inter-laboratory data analysis.

Many issues remain to be resolved, but it is clear that new molecular techniques such as microarray hybridization will have a dramatic impact on toxicology research. In the future, the information gathered from microarray-based hybridization experiments will form the basis for an improved method to assess the impact of chemicals on human and environmental health.

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# Pharmaceutical Proteomics

SANDRA STEINER<sup>a</sup> AND N. LEIGH ANDERSON

*Large Scale Proteomics Corporation, Rockville, Maryland, USA*

**ABSTRACT:** Genomics and proteomics are today well established in drug discovery and, in combination with combinatorial chemistry and high-throughput screening, are helping to bring forward an unprecedented number of potential lead compounds. To avoid the generation of bottlenecks downstream in drug development, increasing pressure is arising to integrate these technologies into the development environment. Proteomics has demonstrated proof-of-concept in toxicology as shown by a number of successful applications in mechanistic toxicology and lead selection. The "technology wave" is now starting to impact the clinical phase of drug development. Expected benefits are optimized clinical trials based on the availability of biologically relevant markers of drug efficacy and safety.

## INTRODUCTION

In recent years, a number of technology developments have had a profound impact on the drug discovery process. The exponential growth in genomics and proteomics capabilities and the subsequent generation of large amounts of novel information have facilitated an unprecedented number of potential new drug targets. Advances in combinatorial chemistry resulting in a nearly unlimited availability of compound libraries, in combination with constant improvements in high-throughput screening (HTS) technologies, have resulted in unprecedented numbers of potential lead compounds. A consequence of this technology-driven acceleration of drug discovery is the creation of bottlenecks downstream in drug development. Thus, increasing pressure is currently arising to integrate genomics and proteomics approaches in the development environment to introduce a similar boost to drug development. The implementation of proteomics in safety assessment has actually advanced beyond the proof-of-concept stage as demonstrated by a significant number of studies performed by our laboratory and by other investigators.

## PROTEOME PROFILING TO OBTAIN INSIGHTS INTO MECHANISMS AND PATHWAYS OF TOXICITY

Insights into molecular mechanisms and knowledge of biological pathways involved in toxic responses are an asset for the interpretation of adverse drug effects and contribute to an accurate risk assessment for humans. There is abundant evidence that links can be established between the up- or downregulation of specific

<sup>a</sup>Address for correspondence: S. Steiner, Large Scale Biology Corporation, 9620 Medical Center Drive, Rockville, MD 20850-3338. Voice: 301-424-5989; fax: 301-762-4892.  
sandra.steiner@lsbc.com

pathways and the morphological manifestation of toxic endpoints, as shown for example with the chemically diverse group of peroxisome proliferating compounds.<sup>1,2</sup> We have studied the liver effects of a set of strong peroxisome proliferators (PP; agonists of the nuclear receptor PPAR $\alpha$ ) and a nonproliferator (an analogue of one of the PPs tested having similar pharmacological potency) in mice and demonstrated three distinct proteome signature patterns.<sup>3</sup> The patterns that could be distinguished corresponded to (i) PPAR $\alpha$  agonist activity, (ii) animal age (30 days difference in age at sacrifice between 5- and 35-day treatment groups), and (iii) action of the non-PP compound by a different mechanism. These results provided support for a unified receptor-based mechanism controlling the main PP response, but demonstrate that individual responsive genes can show quite different dose-response curves. More than 100 proteins showed significant changes following PP treatment, and multiple sensitive markers were identified in this project. Based on the assumption that impairments of biological pathways (as visualized in proteome signature patterns) precede potential morphological manifestations, proteins showing coherent changes following treatment with PPs are likely to be early markers for stimuli that eventually result in proliferation of peroxisomes.

Proteomics was the key to new insights into the molecular mechanisms involved in cyclosporine A (CsA) nephrotoxicity.<sup>4,5</sup> The use of CsA, a potent immunosuppressant, is limited by its kidney toxicity. In kidney proteome patterns from CsA-treated rats, we showed a profound downregulation of the calcium binding protein, calbindin D28, an intracellular calcium buffer and transport protein. Its near absence in the kidneys of CsA-treated animals provides an explanation for the accumulation of calcium in the tubules and consequent tubular toxicity. A subsequent SAR study showed that the downregulation of calbindin was closely associated with immunosuppressant activity<sup>6</sup> and that its downregulation also occurred in humans showing CsA-related nephrotoxicity. Prior to the proteome study, the link in relationship between CsA kidney toxicity and calbindin D28 downregulation was not known.

## PROTEOME PROFILING AND LEAD SELECTION

The selection of lead compounds is a critical step in the drug development process with profound downstream consequences. The selection pressure favors compounds with a wide therapeutic window, as defined by high pharmacological potency in combination with low toxicity. Estimation of therapeutic windows in light of the limited data sets typically available for compounds at the late discovery/early development phase is often similar to guesswork. Thus, information residing in proteome profiles produced from lead candidates can be essential to support compound prioritization decisions.

We used proteome profiling as a basis to select protein markers linked to compound efficacy or toxicity and demonstrated the possibility of using these markers for lead prioritization.<sup>7</sup> A compound studied in this context was SDZ PGU 693, a hypoglycemic agent found to induce hepatocellular hypertrophy in the rat. Liver proteome profiling of treated rats showed the induction of several microsomal proteins, including NADPH cytochrome P-450 reductase and cytochrome b5, indicative of microsomal proliferation and induction of the P-450 enzyme system causing hepatocellular hypertrophy. Decreases were evident in a series of mitochondrial proteins

such as  $F_1$ ATPase-alpha subunit and cytosolic liver fatty acid binding protein suggesting a downregulation of the mitochondrial liver fatty acid metabolism, likely reflecting the pharmacological action of the compound. These data demonstrate that the liver proteome of treated rats revealed protein markers indicative for both SDZ PGU efficacy and toxicity. Markers for both endpoints were selected from these profiles and high-throughput protein assays set up to screen follow-up compounds to assess an estimate of their therapeutic window.

Similarly, efficacy and toxicity markers for "statin"-class cholesterol-lowering compounds were selected using proteome analysis. We found that agents acting to alter blood cholesterol (e.g., the statin HMG-CoA reductase inhibitors, cholestyramine, and high-cholesterol diets) change the abundances of several proteins in rat liver.<sup>8</sup> Most strongly affected is a protein identified as HMG-CoA synthase, a critical enzyme of the cholesterol synthesis pathway. Based on these data, HMG-CoA synthase can be used as an intracellular reporter of the pathway's performance. The statins, but not cholestyramine, also induce a peroxisomal enoyl hydratase-like protein, which was identified as a sensitive marker for peroxisome proliferation (representing in this case an undesirable side effect). The ratio between these efficacy and toxicity markers is different for different members of the marketed statin drugs, indicating their likely pharmacological inequivalence.

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It becomes obvious that the impact of these new technologies will continue downstream into the development process and will extend from the preclinical to the clinic phase. As a consequence, former bottlenecks may disappear and new ones may appear and will need to be addressed. One of these is apparent today and arises from the necessity to obtain proof-of-concept of new drug candidates in humans as early as possible. Phase II and III clinical trials are the most expensive studies in the drug development process and failures at this stage may be fatal (both to the drug and,

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# Pharmaceutical Proteomics

SANDRA STEINER<sup>a</sup> AND N. LEIGH ANDERSON

*Large Scale Proteomics Corporation, Rockville, Maryland, USA*

**ABSTRACT:** Genomics and proteomics are today well established in drug discovery and, in combination with combinatorial chemistry and high-throughput screening, are helping to bring forward an unprecedented number of potential lead compounds. To avoid the generation of bottlenecks downstream in drug development, increasing pressure is arising to integrate these technologies into the development environment. Proteomics has demonstrated proof-of-concept in toxicology as shown by a number of successful applications in mechanistic toxicology and lead selection. The "technology wave" is now starting to impact the clinical phase of drug development. Expected benefits are optimized clinical trials based on the availability of biologically relevant markers of drug efficacy and safety.

## INTRODUCTION

In recent years, a number of technology developments have had a profound impact on the drug discovery process. The exponential growth in genomics and proteomics capabilities and the subsequent generation of large amounts of novel information have facilitated an unprecedented number of potential new drug targets. Advances in combinatorial chemistry resulting in a nearly unlimited availability of compound libraries, in combination with constant improvements in high-throughput screening (HTS) technologies, have resulted in unprecedented numbers of potential lead compounds. A consequence of this technology-driven acceleration of drug discovery is the creation of bottlenecks downstream in drug development. Thus, increasing pressure is currently arising to integrate genomics and proteomics approaches in the development environment to introduce a similar boost to drug development. The implementation of proteomics in safety assessment has actually advanced beyond the proof-of-concept stage as demonstrated by a significant number of studies performed by our laboratory and by other investigators.

## PROTEOME PROFILING TO OBTAIN INSIGHTS INTO MECHANISMS AND PATHWAYS OF TOXICITY

Insights into molecular mechanisms and knowledge of biological pathways involved in toxic responses are an asset for the interpretation of adverse drug effects and contribute to an accurate risk assessment for humans. There is abundant evidence that links can be established between the up- or downregulation of specific

pathways and the morphological manifestation of toxic endpoints, as shown for example with the chemically diverse group of peroxisome proliferating compounds.<sup>1,2</sup> We have studied the liver effects of a set of strong peroxisome proliferators (PP; agonists of the nuclear receptor PPAR $\alpha$ ) and a nonproliferator (an analogue of one of the PPs tested having similar pharmacological potency) in mice and demonstrated three distinct proteome signature patterns.<sup>3</sup> The patterns that could be distinguished corresponded to (i) PPAR $\alpha$  agonist activity, (ii) animal age (30 days difference in age at sacrifice between 5- and 35-day treatment groups), and (iii) action of the non-PP compound by a different mechanism. These results provided support for a unified receptor-based mechanism controlling the main PP response, but demonstrate that individual responsive genes can show quite different dose-response curves. More than 100 proteins showed significant changes following PP treatment, and multiple sensitive markers were identified in this project. Based on the assumption that impairments of biological pathways (as visualized in proteome signature patterns) precede potential morphological manifestations, proteins showing coherent changes following treatment with PPs are likely to be early markers for stimuli that eventually result in proliferation of peroxisomes.

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<sup>a</sup>Address for correspondence: S. Steiner, Large Scale Biology Corporation, 9620 Medical Center Drive, Rockville, MD 20850-3338. Voice: 301-424-5989; fax: 301-762-4892.

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**Subject: RE: [Fwd: Toxicology Chip]****Date: Mon. 3 Jul 2000 08:09:45 -0400****From: "Afshari.Cynthia" <afshari@niehs.nih.gov>****To: "'Diana Hamlet-Cox'" <dianahc@incyte.com>**

You can see the list of clones that we have on our 12K chip at  
<http://manuel.niehs.nih.gov/maps/guest/clonesrch.cfm>

We selected a subset of genes (2000K) that we believed critical to tox response and basic cellular processes and added a set of clones and ESTs to this. We have included a set of control genes (80+) that were selected by the NHGRI because they did not change across a large set of array experiments. However, we have found that some of these genes change significantly after tox treatments and are in the process of looking at the variation of each of these 80+ genes across our experiments.

Our chips are constantly changing and being updated and we hope that our data will lead us to what the toxchip should really be.

I hope this answers your question.

Cindy Afshari

> -----

> From: Diana Hamlet-Cox  
 > Sent: Monday, June 26, 2000 8:52 PM  
 > To: afshari@niehs.nih.gov  
 > Subject: [Fwd: Toxicology Chip]

> Dear Dr. Afshari,

> Since I have not yet had a response from Bill Grigg, perhaps he was not  
 > the right person to contact.

> Can you help me in this matter? I don't need to know the sequences,  
 > necessarily, but I would like very much to know what types of sequences  
 > are being used, e.g., GPCRs (more specific?), ion channels, etc.

> Diana Hamlet-Cox

> ----- Original Message -----

> Subject: Toxicology Chip  
 > Date: Mon, 19 Jun 2000 18:31:48 -0700  
 > From: Diana Hamlet-Cox <dianahc@incyte.com>  
 > Organization: Incyte Pharmaceuticals  
 > To: grigg@niehs.nih.gov

> Dear Colleague:

> I am doing literature research on the use of expressed genes as  
 > pharmacotoxicology markers, and found the Press Release dated February  
 > 29, 2000 regarding the work of the NIEHS in this area. I would like to  
 > know if there is a resource I can access (or you could provide?) that  
 > would give me a list of the 12,000 genes that are on your Human ToxChip  
 > Microarray. In particular, I am interested in the criteria used to  
 > select sequences for the ToxChip, including any control sequences  
 > included in the microarray.

> Thank you for your assistance in this request.

> Diana Hamlet-Cox, Ph.D.  
 > Incyte Genomics, Inc.

> --

> =====

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# Proteomics: a major new technology for the drug discovery process

Martin J. Page, Bob Amess, Christian Rohlff, Colin Stubberfield and Raj Parekh

Proteomics is a new enabling technology that is being integrated into the drug discovery process. This will facilitate the systematic analysis of proteins across any biological system or disease, forwarding new targets and information on mode of action, toxicology and surrogate markers. Proteomics is highly complementary to genomic approaches in the drug discovery process and, for the first time, offers scientists the ability to integrate information from the genome, expressed mRNAs, their respective proteins and subcellular localization. It is expected that this will lead to important new insights into disease mechanisms and improved drug discovery strategies to produce novel therapeutics.

**A**mong the major pharmaceutical and biotechnology companies, it is clearly recognized that the business of modern drug discovery is a highly competitive process. All of the many steps involved are inherently complex, and each can involve a high risk of attrition. The players in this business strive continuously to optimize and streamline the process; each seeking to gain an advantage at every step by attempting to make informed decisions at the earliest stage possible. The desired outcome is to accelerate as many key activities in the drug discovery process as possible. This should pro-

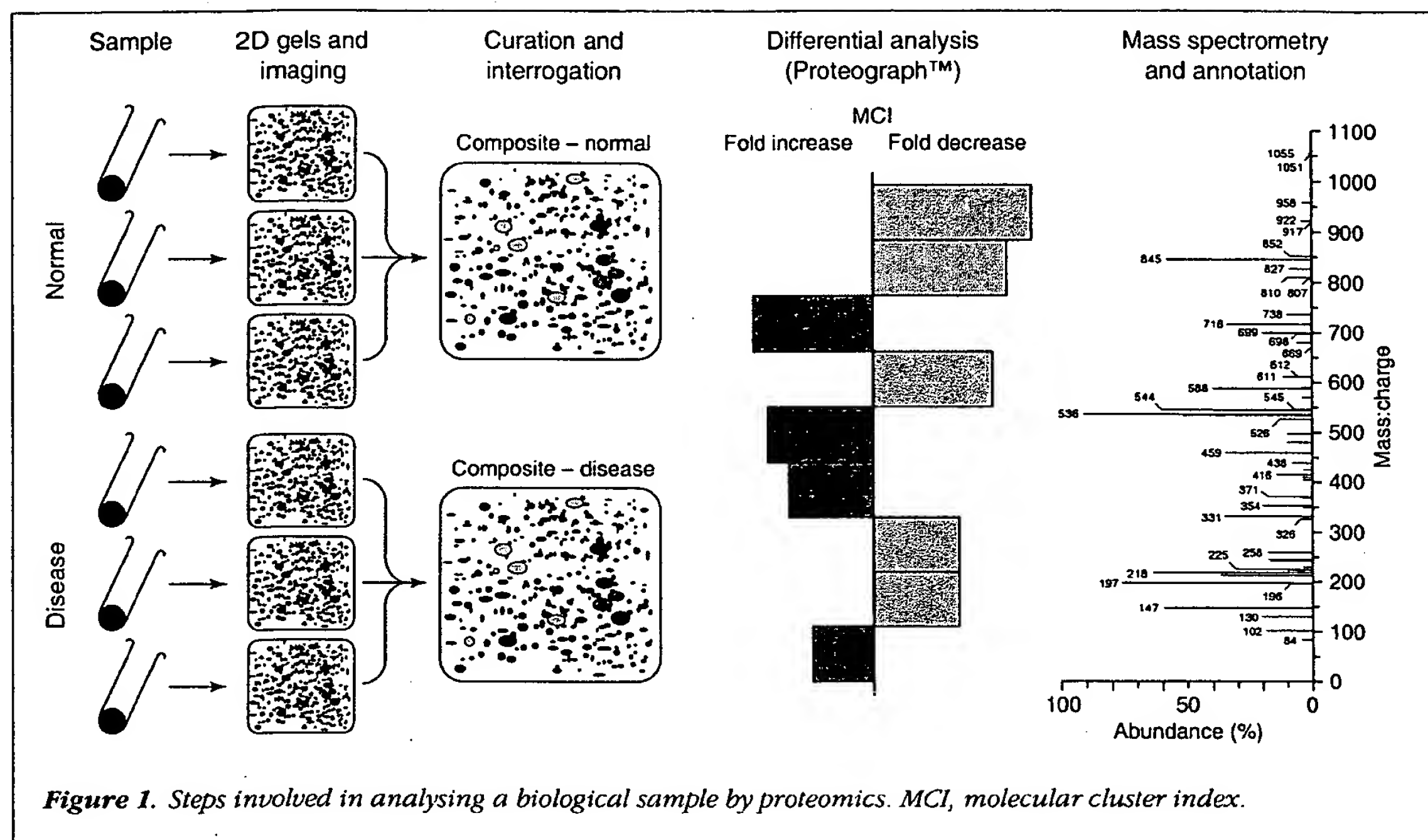
duce a new generation of robust drugs that offer a high probability of success and reach the clinic and market ahead of the competition.

There has been noticeable emphasis over recent years for companies to aggressively review and refine their strategies to discover new drugs. Central to this has been the introduction and implementation of cutting-edge technologies. Most, if not all, companies have now integrated key technology platforms that incorporate genomics, mRNA expression analysis, relational databases, high-throughput robotics, combinatorial chemistry and powerful bioinformatics. Although it is still early days to quantify the real impact of these platforms in clinical and commercial terms, expectations are high, and it is widely accepted that significant benefits will be forthcoming. This is largely based on data obtained during preclinical studies where the genomic<sup>1,2</sup> and microarray<sup>3,4</sup> technologies have already proved their value.

However, there are several noteworthy outcomes that result from this. Many comments are voiced that scientists armed with these technologies are now commonly faced with data overload. Thus, in some instances, rather than facilitating the decision process, the accumulation of more complex data points, many with unknown consequences, can seem to hinder the process. Also, most drug companies have simultaneously incorporated very similar components of the new technology platforms, the consequence being that it is becoming difficult yet again to determine where a clear competitive advantage will arise. Finally, in recent years, largely as a result of the accessibility of the technologies, there has been an overwhelming emphasis placed on genomic and mRNA data rather than on protein

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Martin J. Page\*, Bob Amess, Christian Rohlff, Colin Stubberfield and Raj Parekh, Oxford GlycoSciences, 10 The Quadrant, Abingdon Science Park, Abingdon, Oxfordshire, UK OX14 3YS. \*tel: +44 1235 543277, fax: +44 1235 543283, e-mail: martin.page@ogs.co.uk



analysis. It is important to remember that proteins dictate biological phenotype – whether it is normal or diseased – and are the direct targets for most drugs.

#### Proteomics: new technology for the analysis of proteins

It is now timely to recognize that complementary technology in the form of high-throughput analysis of the total protein repertoire of chosen biological samples, namely proteomics, is poised to add a new and important dimension to drug discovery. In a similar fashion to genomics, which aims to profile every gene expressed in a cell, proteomics seeks to profile every protein that is expressed<sup>5-7</sup>. However, there is added information, since proteomics can also be used to identify the post-translational modifications of proteins<sup>8</sup>, which can have profound effects on biological function, and their cellular localization. Importantly, proteomics is a technology that integrates the significant advances in two-dimensional (2D) electrophoretic separation of proteins, mass spectrometry and bioinformatics. With these advances it is now possible to consistently derive proteomes that are highly reproducible and suitable for interrogation using advanced bioinformatic tools.

There are many variations whereby different laboratories operate proteomics. For the purpose of this review, the

process used at Oxford GlycoSciences (OGS), which uses an industrial-scale operation that is integral to its drug discovery work, will be described. The individual steps of this process, where up to 1000 2D gels can be run and analysed per week, are summarized in Fig. 1. The incoming samples are bar coded and all information relevant to the sample is logged into a Laboratory Information Management System (LIMS) database. There can be a wide range in the type of samples processed, as applicable to individual steps in the drug discovery pipeline, and these will be mentioned later. The samples are separated according to their charge (pI) in the first dimension, using isoelectric focusing, followed by size (MW) using SDS-PAGE in the second dimension. Many modifications have been made to these steps to improve handling, throughput and reproducibility. The separated proteins are then stained with fluorescent dyes which are significantly more sensitive in detection than standard silver methods and have a broader dynamic range. The image of the displayed proteins obtained is referred to as the proteome, and is digitally scanned into databases using proprietary software called ROSETTA™. The images are subsequently curated, which begins with the removal of any artefacts, cropping and the placement of pI/MW landmarks. The images from replicate images are then aligned and matched to one

another to generate a synthetic composite image. This is an important step, as the proteome is a dynamic situation, and it captures the biological variation that occurs, such that even orphan proteins are still incorporated into the analysis.

By means of illustration, Fig. 1 shows the process whereby proteomes are generated from normal and disease samples and how differentially expressed proteins are identified. The potential of this type of analysis is tremendous. For example, from a mammalian cell sample, in excess of 2000 proteins can typically be resolved within the proteome. The quality of this is shown in Fig. 2, which shows representative proteomes from three diverse biological sources: human serum, the pathogenic fungus *Candida albicans* and the human hepatoma cell line Huh7.

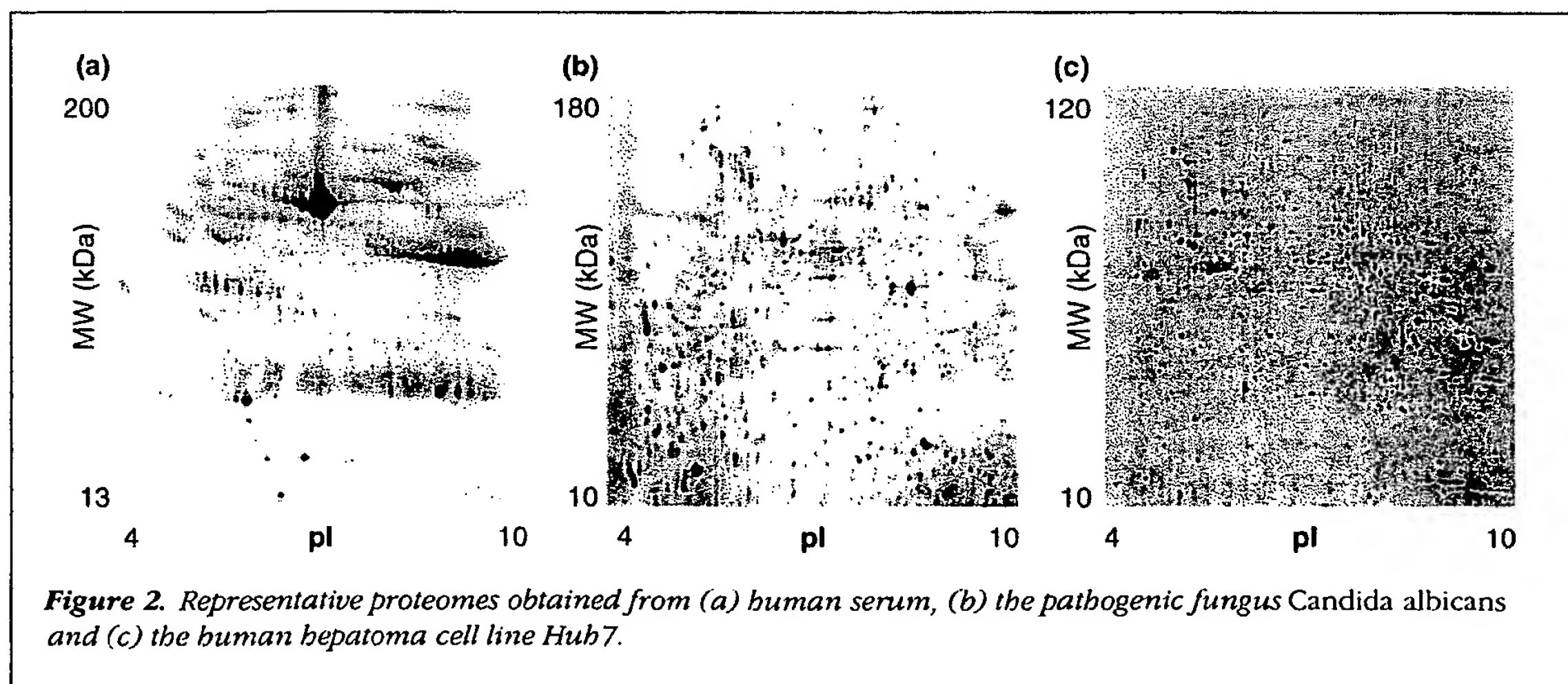
#### Use of proteomics to identify disease specific proteins

In most cases, the drug discovery process is initiated by the identification of a novel candidate target – almost always a protein – that is believed to be instrumental in the disease process. To date, there is a variety of means whereby drug targets have been forthcoming. These include molecular, cellular and genomic approaches, mostly centred upon DNA and mRNA analysis. The gene in question is isolated, and expression and characterization of its coded protein product – i.e. the drug target – is invariably a secondary event.

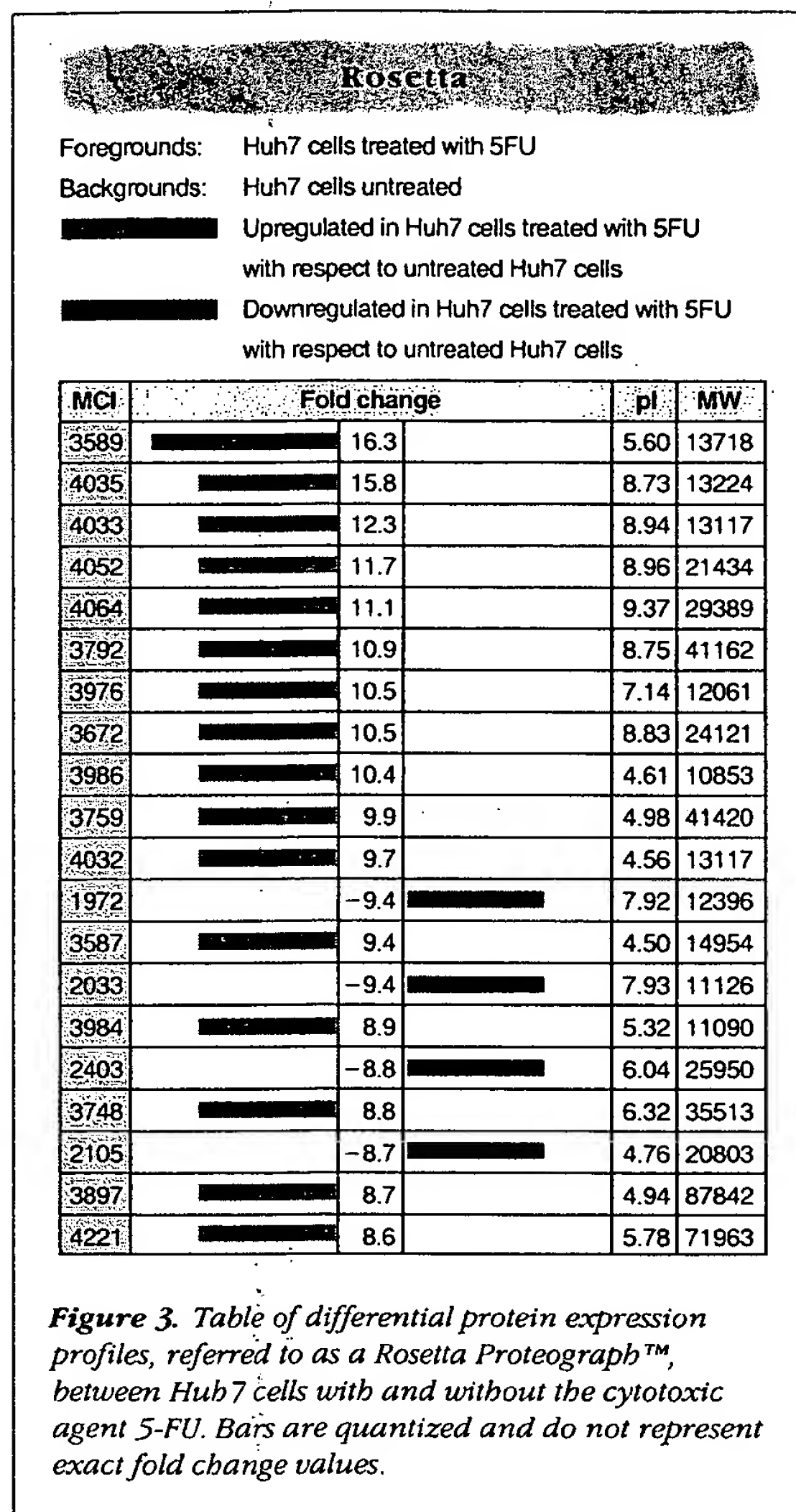
With the proteomic approach, the starting point is at the other end of the 'telescope'. Here there is direct and im-

mediate comparison of the proteomes from paired normal and disease materials. Examples of these pairs are: (1) purified epithelial cell populations derived from human breast tumours, matched to purified normal populations of human breast epithelial cells, and (2) the invading pathogenic hyphal form of *C. albicans*, matched to the non-invading yeast form of *C. albicans*. When the proteome images from each pair are aligned, the Proteograph™ software is able to rapidly identify those proteins (each referenced as having a unique molecular cluster index, or MCI) that are either unique, or those that are differentially expressed. Thus, the Proteograph output from this analysis is both qualitative and quantitative.

Proteograph analysis for a particular study can also be undertaken on any number of samples. For example, one might compare anything from a few to several hundred preparations or samples, each from a normal and disease counterpart, and have these analysed in a single Proteograph study. In this way, it is possible to assign strong statistical confidence to the data and in some instances to identify specific subpopulations within the input biological sources. This feature will become increasingly significant in the near future, and there is a clear synergy here whereby proteomics can work closely with pharmacogenomic approaches to stratify patient populations and achieve effective targeted care for the patient. Whatever the source of the materials, the net output of Proteograph analysis is immediate identification of disease specific proteins. This is shown in Fig. 3, which shows the results of a proteograph obtained by comparing untreated human hepatoma cells with cells following exposure to a clinical







cytotoxic agent. In this instance, only the top 20 differentially expressed MCIs are shown, but the readout would normally extend to a defined cut-off value, typically a two-fold or greater difference in expression levels, determined by the user.

In a typical analysis involving disease and normal mammalian material, in which each proteome would have ~2000 protein features each assigned an MCI, the proteograph might identify somewhere in the region of 50–300 MCIs that are unique or differentially expressed. To capitalize rapidly on these data, at OGS a high-throughput

mass spectrometry facility coupled to advanced databases to annotate these MCIs as individual proteins is applied. As these are all disease specific proteins, each could represent a novel target and/or a novel disease marker. The process becomes even more powerful when a panel of features, rather than individual features, are assigned. The relevance of this is apparent when one considers that most diseases, if not all, are multifactorial in nature and arise from polygenic changes. Rather than analysing events in isolation, the ability to examine hundreds or thousands of events simultaneously, as shown by proteomics, can offer real advantages.

#### Identification and assignment of candidate targets

The rapid identification and assignment of candidate targets and markers represents a huge challenge, but this has been greatly facilitated by combining the recent advances made in proteomics and analytical mass spectrometry<sup>9</sup>. Using automated procedures it is now possible to annotate proteins present in femtomole quantities, which would depict the low abundance class of proteins. The process of annotation is similarly aided by the quality and richness of the sequence specific databases that are currently available, both in the public domain and in the private sector (e.g. those supplied by Incyte Pharmaceuticals). In this respect, the advances in proteomics have benefited considerably from the breakthroughs achieved with genomics.

From an application perspective, cancer studies provide a good opportunity whereby proteomics can be instrumental in identifying disease specific proteins, because it is often feasible to obtain normal and diseased tissue from the same patient. For example, proteomic studies have been reported on neuroblastomas<sup>10</sup>, human breast proteins from normal and tumour sources<sup>11–13</sup>, lung tumours<sup>14</sup>, colon tumours<sup>15</sup> and bladder tumours<sup>16</sup>. There are also proteomic studies reported within the cardiovascular therapeutic area, in which disease or response proteins are identified<sup>17,18</sup>.

Genomic microarray analysis can similarly identify unique species or clusters of mRNAs that are disease specific. However, in some instances, there is a clear lack of correlation between the levels of a specific mRNA and its corresponding protein (Ref. 19, Gypi, S.P. *et al.*, submitted). This has now been noted by many investigators and reaffirms that post-transcriptional events, including protein stability, protein modification (such as phosphorylation, glycosylation, acylation and methylation) and cell localization, can constitute major regulatory steps. Proteomic analysis captures all of these steps and can therefore provide unique and valuable information independent from, or complementary to, genomic data.



**Proteomics for target validation and signal transduction studies**

The identification of disease specific proteins alone is insufficient to begin a drug screening process. It is critical to assign function and validation to these proteins by confirming they are indeed pivotal in the disease process. These studies need to encompass both gain- and loss-of-function analyses. This would determine whether the activity of a candidate target (an enzyme, for example), eliminated by molecular/cellular techniques, could reverse a disease phenotype. If this happened, then the investigator would have increased confidence that a small-molecule inhibitor against the target would also have a similar effect. The proposal of candidate drug targets is often not a difficult process, but validating them is another matter. Validation represents a major bottleneck where the wrong decision can have serious consequences<sup>20</sup>.

Proteomics can be used to evaluate the role of a chosen target protein in signal transduction cascades directly relevant to the disease. In this manner, valuable information is forthcoming on the signalling pathways that are perturbed by a target protein and how they might be corrected by appropriate therapeutics. Techniques that are well established in one-dimensional protein studies to investigate signalling pathways, such as western blotting and immunoprecipitation, are highly suited to proteomic applications. For example, the proteomes obtained can be blotted onto membranes and probed with antibodies against the target protein or related signalling molecules<sup>21-23</sup>. Because proteomics can resolve >2000 proteins on a single gel, it is possible to derive important information on specific isoforms (such as glycosylated or phosphorylated variants) of signalling molecules. This will result in characterization of how they are altered in the disease process. Western immunoblotting techniques using high-affinity antibodies will typically identify proteins present at ~10 copies per cell (~1.7 fmol); this is in contrast to the best fluorescent dyes currently available that are limited to imaging proteins at 1000 or more copies per cell. The level of sensitivity derived by these applications will greatly facilitate interpretation of complex signalling pathways and contribute significantly to validation of the target under study.

**Immunoprecipitation studies**

Similarly, immunoprecipitation studies are another useful way to exploit the resolving power of proteomics<sup>24,25</sup>. In this instance, very large quantities of protein (e.g. several milligrams) can be subjected to incubation with antibodies against chosen signalling molecules. This allows high-affin-

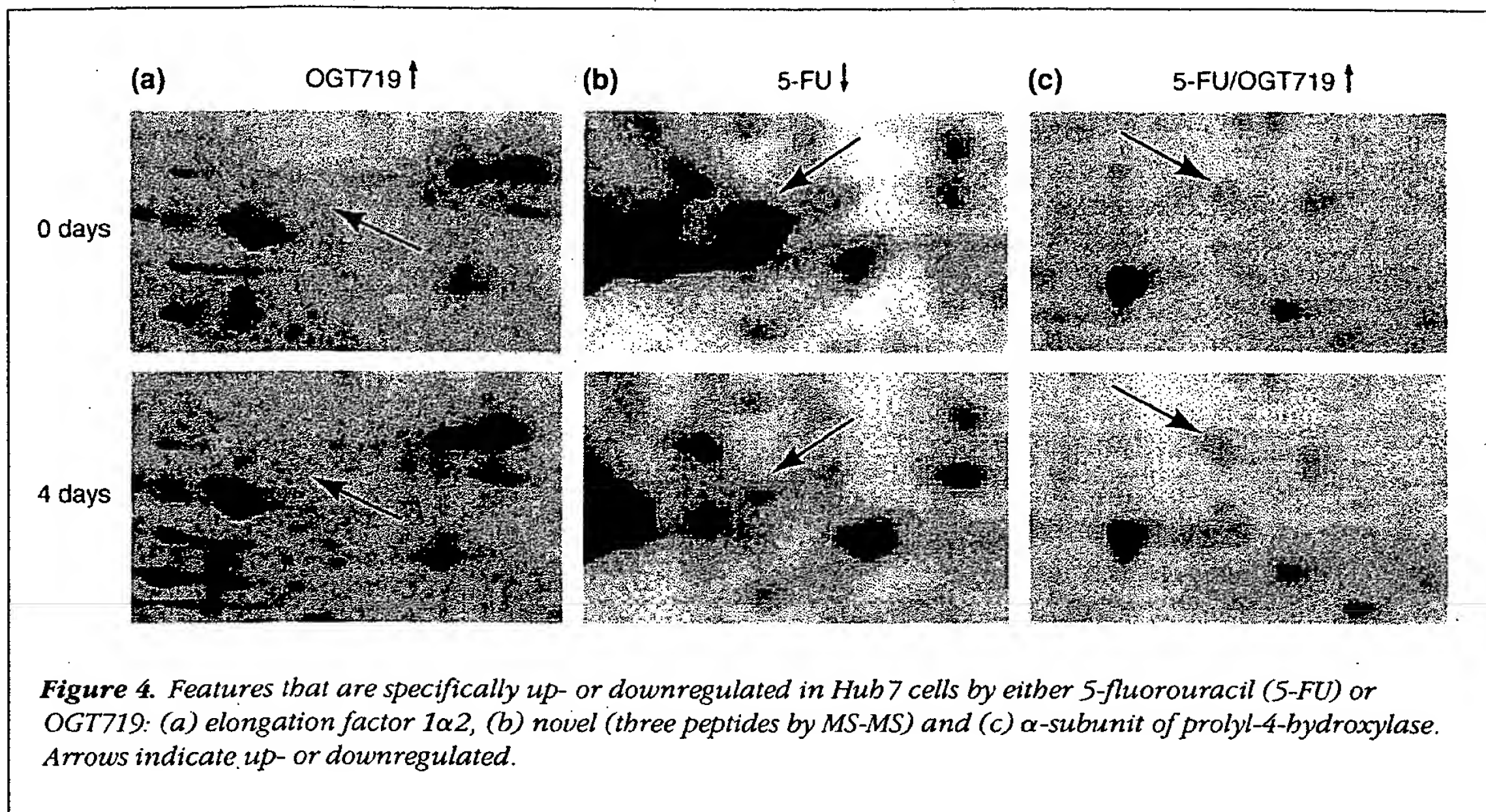
ity capture of these proteins, which can subsequently be eluted and electrophoresed on a 2D gel to provide a high-resolution proteome of a specific subset of proteins. Detection by blot analysis allows the identification of extremely small amounts of defined signalling molecules. Again, the different isoforms of even very low abundance proteins can be seen, and, very importantly, the technique allows the investigator to identify multiprotein complexes or other proteins that co-precipitate with the target protein. These coassociating proteins frequently represent signalling partners for the target protein, and their identification by mass spectrometry can lead to invaluable information on the signalling processes involved.

The depth of signal transduction analysis offered by proteomics, and the utility for target validation studies, can be extended even further by applying cell fractionation studies<sup>26-28</sup>. By purifying subcellular fractions, such as membrane, nuclear, organelle and cytosolic, it is possible to assign a localization to proteins of interest and to follow their trafficking in a cell. Enrichment of these fractions will also allow much higher representation of low abundance proteins on the proteome. Their detection by fluorescent dyes or immunoblot techniques will lead to the identification of proteins in the range of 1-10 copies per cell, putting the sensitivity on a par with genomic approaches.

These signal transduction analyses can be of additional value in experiments where inhibitors derived from a screening programme against the target are being evaluated for their potency and selectivity. The inhibitors can encompass small molecules, antisense nucleic acid constructs, dominant-negative proteins, or neutralizing antibodies microinjected into cells. In each case, proteome analysis can provide unique data in support of validation studies for a chosen candidate drug target.

**Proteomics and drug mode-of-action studies**

Once a validated target is committed to a screening regimen to identify and advance a lead molecule, it is important to confirm that the efficacy of the inhibitor is through the expected mechanism. Such mode-of-action studies are usually tackled by various cell biological and biochemical methods. Proteomics can also be usefully applied to these studies and this is illustrated below by describing data obtained with OGT719. This is a novel galactosyl derivative of the cytotoxic agent 5-fluorouracil (5-FU), which is currently being developed by OGS for the treatment of hepatocellular carcinoma and colorectal metastases localized in the liver. The premise underpinning the design and rationale of OGT719 was to derive a 5-FU prodrug capable



of targeting, and being retained in, cells bearing the asialoglycoprotein receptor (ASGP-r), including hepatocytes<sup>29</sup>, hepatoma Huh7 cells<sup>30</sup> and some colorectal tumour cells<sup>31</sup>. The growth of the human hepatoma cell line Huh7 is inhibited by 5-FU or by OGT719. If the inhibition by OGT719 were the result of uptake and conversion to 5-FU as the active component, then it would be expected that Huh7 cells would show similar proteome profiles following exposure to either drug.

To examine these possibilities, we conducted an experiment taking samples of Huh7 cells that had been treated with IC<sub>50</sub> doses of either OGT719 or 5-FU. Total cell lysates were prepared and taken through 2D electrophoresis, fluorescence staining, digital imaging and Proteograph analysis. To facilitate the interpretation of the data across all of the 2291 features seen on the proteomes, drug-induced protein changes of fivefold or greater, identified by the Proteograph, were analysed further. Interestingly, from this analysis 19 identical proteins were changed fivefold or more by both drugs, strongly suggesting similarities in the mode of action for these two compounds.

Thus, from very complex data involving >2000 protein features, using proteomics it is possible to analyse quantitatively and qualitatively each protein during its exposure to drugs. The biologist is now able to focus a series of further studies specifically on an enriched subset of proteins.

Figure 4 shows highlighted examples of the selected areas of the proteome where some of these identified proteins in the above study are altered in response to either or both drugs.

Several of the proteins identified above as being modulated similarly by 5-FU or OGT719 in Huh7 cells were subjected to tandem mass-spectrometric analysis for annotation. Some of these, such as the nuclear ribosomal RNA-binding protein<sup>32</sup>, can be placed into pyrimidine pathways or related cell cycle/growth biochemical pathways in which 5-FU is known to act.

To attribute further significance to the proteome mode-of-action studies with OGT719, another cell line, the rat sarcoma HSN, was used. Growth of these cells is inhibited by 5-FU, but they are completely refractory to OGT719; notably they lack the ASGP-r, which might explain this finding (unpublished). For our proteome studies, HSN cells were treated with 5-FU or OGT719 over a time course of one, two and four days. At each time point, cells were harvested and processed to derive proteomes and Proteographs. As before, we purposely focused on those proteins that increased or decreased by fivefold or more. In this instance, there were no proteins co-modulated by the two drugs. This is perhaps to be expected, given that the HSN cells are killed by 5-FU and yet are refractory to OGT719.



*Clear potential*

The above is just an example of how proteomics can be used to address the mode of action of anticancer drugs. The potential of this approach is clear, and one can envisage situations where it will be profitable to compare the proteomes of cells in which the drug target has been eliminated by molecular knockout techniques, or with small-molecule inhibitors believed to act specifically on the same target. In addition to using proteomics to examine the action of drugs, it is also possible to use this approach to gauge the extent of nonspecific effects that might eventually lead to toxicity. For instance, in the example used above with HSN cells treated with OGT719, although cell growth was not affected, the levels of several specific proteins were changed. Further investigation of these proteins and the signalling pathways in which they are involved could be illuminating in predicting the likelihood or otherwise of long-term toxicity.

**Use of proteomics in formal drug toxicology studies**

A drug discovery programme at the stage where leads have been identified and mode-of-action studies are advanced, will proceed to investigate the pharmacokinetic and toxicology profile of those agents. These two parameters are of major importance in the drug discovery process, and many agents that have looked highly promising from *in vitro* studies have subsequently failed because of insurmountable pharmacokinetic and/or toxicity problems *in vivo*. Whereas the pharmacokinetic properties of a molecule can now be characterized quickly and accurately, toxicity studies are typically much longer and more demanding in their interpretation.

The ability to achieve fast and accurate predictions of toxicity within an *in vivo* setting would represent a big step forward in accelerating any drug discovery programme. Toxicity from a drug can be manifested in any organ. However, because the liver and kidney are the major sites in the body responsible for metabolism and elimination of most drugs, it is informative to examine these particular organs in detail to provide early indications about events that might result in toxicity.

The basis for most xenobiotic metabolizing activity is to increase the hydrophilicity of the compound and so facilitate its removal from the body. Most drugs are metabolized in the liver via the cytochrome P450 family of enzymes, which are known to comprise a total of ~200 different members<sup>33,34</sup>, encompassing a wide array of overlapping specificities for different substrates. In addition to clearance, they also play a major role in metabo-

lism that can lead to the production and removal of toxic species, and in some instances it is possible to correlate the ability or failure to remove such a toxin with a specific P450 or subgroup.

*Unique P450 profiles*

Each individual person will have a slightly different P450 profile, largely from polymorphisms and changes in expression levels, although other genetic and environmental factors aside from P450 also need to be taken into consideration. A significant amount of research is currently being directed towards this field – known as pharmacogenomics – with the aim of predicting how a patient will respond to a drug, as determined by their genetic make-up<sup>35–37</sup>. The marked variation of individuals in their ability to clear a compound can be one of the key factors in deciding the overall pharmacokinetic profile of a drug. Not only will this have a bearing on the likelihood of a patient responding to a treatment, but it will also be a factor in determining the possibility of their experiencing an adverse effect.

Many pharmaceutical companies are already employing genomic approaches, involving P450 measurements, as a key step in their assessment of the toxicological profile of a candidate drug and therefore of its suitability, or otherwise, to be considered for human clinical trials. There are limits to this approach, however. Whereas the P450 mRNA profiling can predict with some accuracy the likely metabolic fate of a drug, it will not provide information on whether the metabolites would subsequently lead to toxicity. Besides the patient-to-patient differences in steady-state levels of the P450s, there are also characteristic induction responses of these enzymes to some drugs. Moreover, as there can be some doubt over the correlation of mRNA levels and the corresponding protein levels, there is scope for misinterpretation of the results and hence real advantages to be gained from a proteome approach. In both instances, the ability to examine entire proteome profiles, including the P450 proteins, will be a significant advantage in understanding and predicting the metabolism and toxicological outcome of drugs.

In addition to direct organ and tissue studies, the serum, which collects the majority of toxicity markers released from susceptible organs and tissues throughout the entire body, can be utilized. Serum is rich in nuclease activity and, as pharmacogenomics is not suited to deal with these samples, valuable markers of toxicity could go undetected. However, by using proteomics for these types of analyses, serum markers (and clusters thereof) are now accessible for evaluation as indicators of toxicity.

### Pharmacoproteomics

Proteomics can thus be used to add a new sphere of analysis to the study of toxicity at the protein level, and in the era of '-omics' there is a case to be made to adopt the term 'Pharmacoproteomics™'. Animals can be dosed with increasing levels of an experimental drug over time, and serum samples can be drawn for consecutive proteome analyses. Using this procedure, it should be possible to identify individual markers, or clusters thereof, that are dose related and correlate with the emergence and severity of toxicity. Markers might appear in the serum at a defined drug dose and time that are predictive of early toxicity within certain organs and if allowed to continue will have damaging consequences. These serum markers could subsequently be used to predict the response of each individual and allow tailoring of therapy whereby optimal efficacy is achieved without adverse side effects being apparent. This application can obviously extend to tracking toxicity of drugs in clinical trials where serum can be readily drawn and analysed. Surrogate markers for drug efficacy could also be detected by this procedure and could facilitate the challenge of identifying patient classes who will respond favourably to a drug and at what dosage.

### Conclusions

By contrast to the agents administered to patients in clinical wards, the process of drug discovery is not a prescriptive series of steps. The risks are high and there are long timelines to be endured before it is known whether a candidate drug will succeed or fail. At each step of the drug discovery process there is often scope for flexibility in interpretation, which over many steps is cumulative. The pharmaceutical companies most likely to succeed in this environment are those that are able to make informed accurate decisions within an accelerated process.

The genomics revolution has impacted very positively upon these issues and now has a powerful new partner in proteomics. The ability to undertake global analysis of proteins from a very wide diversity of biological systems and to interrogate these in a high-throughput, systematic manner will add a significant new dimension to drug discovery. Each step of the process from target discovery to clinical trials is accessible to proteomics, often providing unique sets of data. Using the combination of genomics and proteomics, scientists can now see every dimension of their biological focus, from genes, mRNA, proteins and their subcellular localization. This will greatly assist our understanding of the fundamental mechanistic basis of human disease and allow new improved and speedier drug discovery strategies to be implemented.

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